Studies on the chemical nature of procoagulant sites on phospholipid vesicles and platelet membranes

STUDIES ON THE CHEMICAL NATURE OF PROCOAGULANT SITES ON PHOSPHOLIPID VESICLES AND PLATELET MEMBRANES

Proefschrift

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CONTENTS

ABBREVIATIONS

Dansyl-Glu-Gly-Arg-CH_2Cl	Dansyl-L-glutamyl-glycyl-L-arginine chloromethyl ketone dihydrochloride
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminotetraacetic acid
F1	Prothrombin fragment 1
F1.2	Prothrombin fragment 1.2
Gla	γ-carboxyglutamic acid
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
I2581	N-dansyl-(p-guanidino)phenylalanine-piperidide hydrochloride
M_r	Relative molecular weight
PA	1,2-dioleoyl-sn-glycero-3-phosphate
PC	1,2-dioleoyl-sn-glycero-3-phosphocholine
PG	1,2-dioleoyl-sn-glycero-3-phosphoglycerol
PI	1,2-dioleoyl-sn-glycero-3-phosphoinositol
PLac	1,2-dioleoyl-sn-glycero-3-phospho-β-lactate
PS	1,2-dioleoyl-sn-glycero-3-phosphoserine
Phe-Pro-Arg-CH_2Cl	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride
phospholipase A_2	Phospatide 2-acylhydrolase
p-NPGB	p-nitrophenyl-p'-guanidinobenzoate
QAE	Quaternary aminoethyl
RVV-X	Factor X activator purified from Russell's Viper venom
SDS	Sodium dodecyl sulphate
SP	Sulfopropyl
STI	Soybean trypsin inhibitor
S2222	N-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-p-nitroanilide hydrochloride
S2238	H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride
S2337	N-benzoyl-L-isoleucyl-L-glutamyl(piperidyl) glycyl-L-arginine-p-nitroanilide hydrochloride

CHAPTER I

INTRODUCTION

In blood several components are present that provide for coagulant and anticoagulant mechanisms. A delicate balance between these mechanisms prevails, which normally is in favour of anticoagulation to keep the blood fluid. Nevertheless, procoagulant reactions are temporarily triggered at a site of vessel wall damage to produce a hemostatic plug that arrests bleeding. A permanent imbalance to either side, however, has rather profound implications: bleeding disorders, on the one hand, or thrombosis and cardiovascular disease on the other. Understanding the hemostatic processes is necessary for active prevention of hemostatic malfunctions and for the development of rational therapeutical approaches. Furthermore, studies of hemostasis, with its complicated chemical reactions and interactions, will extend our insights in the fundamentals of protein and lipid biochemistry. The next paragraphs will give a short general introduction to hemostasis and discuss in more detail those aspects of the hemostatic process that are subject of this thesis.

The process of blood coagulation. Hemostasis covers several sequential steps which can be divided into two pathways as is shown in a simplified scheme in Fig. 1. Platelets are small, sticky, discoid blood cells, which adhere to exposed collagen fibres at a site of vessel wall injury. Here they become activated, change shape and release several components, some of which may stimulate other platelets from the blood stream to adhere, and thus form a platelet aggregate (1). Simultaneously the damaged vessel wall initiates the coagulation cascade: clotting factors, circulating in the blood as inactive zymogens, become successively activated (2). This finally leads to the formation of thrombin, a proteolytic enzyme, which plays a key role in hemostasis. One of the functions of thrombin is the enzymatic conversion of fibrinogen to fibrin. The fibrin monomers that are formed crosslink to produce polymer threads, which strengthen the platelet aggregate to render the hemostatic plug. Thrombin, like collagen, also has

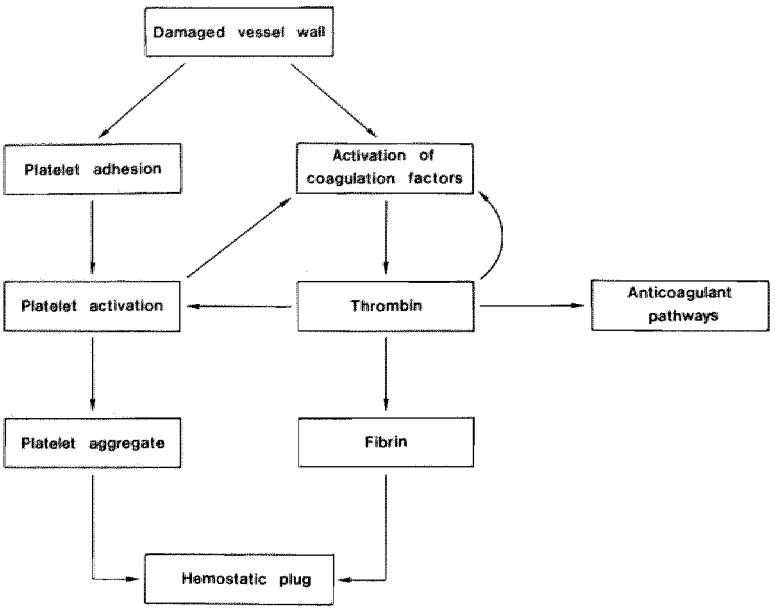

Fig. 1. **Schematic representation of the hemostatic process**

the ability to induce platelet activation and aggregation. Activated platelets expose a procoagulant surface to which the coagulation factors may bind. Thus, platelets promote the interaction and activation of the clotting factors (3). Furthermore, thrombin activates in a feedback reaction Factors V and VIII, and so accelerates its own formation. A fourth important property of thrombin is that it initiates anticoagulant pathways (4). This leads to the control of the hemostatic process and finally to the restoration of the subtile interplay of coagulant and anticoagulant reactions.

From this, it is clear that thrombin is a central component in blood coagulation. The mechanism of its generation is therefore of special interest.

The conversion of prothrombin to thrombin. Thrombin is formed due to partial proteolysis of the zymogen prothrombin by Factor Xa (5), a serine protease that originates from its precursor Factor X earlier in the coagulation cascade. Prothrombin activation by Factor Xa alone is a very

slow process. The reaction is, however, enormously accelerated in the presence of negatively charged phospholipids, calcium ions and the non-enzymatic protein cofactor Va (6). The phospholipids provide a membrane surface to which Factor Xa, Factor Va and prothrombin bind (6,7), and thus promote the interactions of these coagulation factors, while Factor Va has a major effect on the catalytic efficiency of Factor Xa (see below).

In the last two decennia much information has been obtained regarding the proteins involved in prothrombin activation. The proteins have been purified to homogeneity and structural and functional properties have been established. Fig. 2 shows some of the characteristics of these proteins.

Factor Xa is a glycoprotein, with a molecular weight of 45,000, that consists of two disulfide-linked polypeptide chains (8,9). The light chain (M_r 18,000) of Factor Xa contains γ-carboxyglutamic acid (Gla) residues (10), which are essential for the calcium-dependent binding of Factor Xa to procoagulant phospholipid surfaces (11,12). The heavy chain (M_r 27,000) of Factor Xa contains the active site region of the molecule and has a high degree of amino acid sequence homology with trypsin (13).

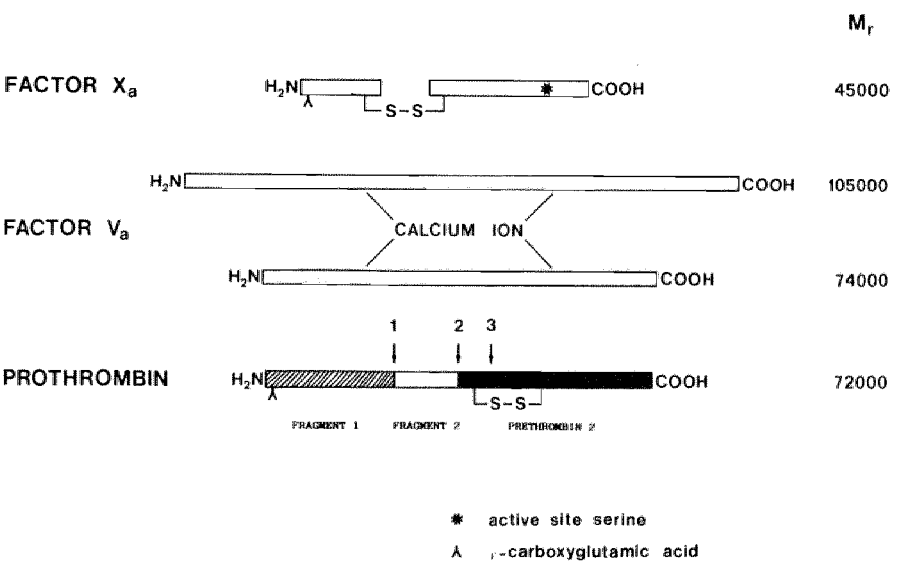

Fig. 2. Proteins participating in prothrombin activation

Factor Va is a glycoprotein with a molecular weight of 180,000 that is composed of a heavy chain of M_r 105,000 and a light chain of M_r 74,000 (14). The two chains are non-covalently associated through a calcium bridge. Factor Va is thought to bind to negatively charged phospholipids through the light chain (15,16). Details of the binding characteristics are not known, although both hydrophobic (15) and ionic (16) interactions have been reported.

Prothrombin is a single chain glycoprotein with a molecular weight of 72,000, which consists of three functional domains. The fragment 1 region contains the Gla residues (17), which are thought to be directly involved in the calcium-mediated binding of prothrombin to negatively charged phospholipids. A number of studies indicate, however, that some of the Gla's may also be involved in a calcium-induced conformational change of the prothrombin molecule, that is essential for biological activity (18,19,20). The fragment 2 region is the part of the protein that is thought to interact with the protein cofactor Va (21). It is followed by the so-called prethrombin 2 region. From this region the trypsin-like serine protease thrombin is actually generated after limited proteolysis of prothrombin by Factor Xa (22).

Three bonds in the prothrombin molecule are susceptible for proteolytic cleavage. Cleavage at bond 1 (Arg_{156}-Ser_{157}) is catalyzed by thrombin, rendering fragment 1 and prethrombin 1. Since prethrombin 1 lacks the membrane binding domain of prothrombin it is a poor substrate for membrane-bound Factor Xa and thrombin therefore imposes a negative feedback on its own formation.

Factor Xa can hydrolyse the Arg_{274}-Thr_{275} bond (site 2) and the Arg_{323}-Ile_{324} bond (site 3) of prothrombin. Since both peptide bonds have to be cleaved during the conversion of prothrombin into thrombin, there are actually two possible pathways of prothrombin activation (Fig. 3). By route A, first site 2 is cleaved, rendering the activation peptide fragment 1.2 and prethrombin 2, a catalytically inactive intermediate. Subsequently Factor Xa splits prethrombin 2 at site 3 to give thrombin. In pathway B, the reversed order of bond cleavage is followed, yielding the intermediate meizothrombin, which has its catalytic site exposed and is fully active towards small synthetic substrates, but which has a diminished clotting activity (23). Depending on the reaction conditions chosen both

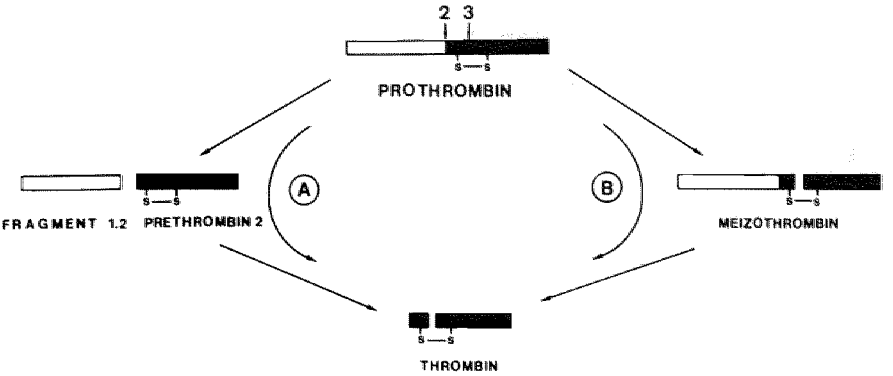

Fig. 3. Pathways of prothrombin activation

intermediates prethrombin 2 (24) and meizothrombin (25) can be generated, indicating that thrombin may be formed via both pathways.

The function of phospholipids in prothrombin activation. Kinetic experiments have shown that phospholipids exert their effect on the rate of thrombin formation by a drastic decrease of the Km for prothrombin to levels below the plasma concentration (26). Moreover, phospholipids enhance the binding affinity of Factor Xa for Factor Va, which form a tight, lipid-bound enzymatic complex called prothrombinase (27,28).

Two models have been proposed to explain the effects of phospholipids on the Km for prothrombin conversion. In the so-called "bound substrate" model, phospholipids are viewed as a surface to which the enzyme Factor Xa and the substrate prothrombin bind, thereby raising their local concentrations and thus promoting their ability to interact and react with each other (26,29,30). Lipid-bound Factor Xa converts lipid-bound prothrombin and the increased prothrombin concentration at the lipid-water interface explains the decrease of the observed Km. The model was supported by the observation that the density of prothrombin at the membrane surface appeared to determine the reaction kinetics of prothrombin activation (26).

Nelsestuen proposed the "free substrate" model (31), in which prothrombinase is depicted as a dissociable three-component enzyme (Factor Xa-Factor Va-phospholipid) that converts soluble prothrombin. He suggested that membranes impose their effect on the Km for prothrombin by virtue of

the additive free energies of prothrombin-phospholipid and prothrombin-Factor Va interactions, which occur at the active site of prothrombinase (31,32). Experiments with membranes that have a low binding affinity for prothrombin sustain these views (33,34). The Km for prothrombin activation on such membranes in the presence of Factor Va was much lower than could be expected from the lipid-binding parameters of prothrombin. Van Rijn et al. (33) demonstrated that prothrombin activation with or without Factor Va cannot be described by one model. In the presence of Factor Va free prothrombin appears to govern prothrombinase activity, while in the absence of Factor Va, the effect of phospholipids on the observed Km is qualitatively best explained in the bound-substrate model.

The function of Factor Va in prothrombin activation. The effect of Factor Va on the kinetic parameters of prothrombin activation has been the subject of a number of studies (26,27,33,34). Factor Va dramatically increases the Vmax of prothrombin conversion (26,27), while a decrease of the Km for prothrombin is also observed, a feature which becomes most apparent on membranes with a low binding affinity for prothrombin (33,34).

The effect on the Vmax appears to be threefold. Factor Va raises the k_{cat} of thrombin formation more than 1000 times. Whether it exerts this effect by making Factor Xa a better enzyme, or by modifying the prothrombin molecule, to make it more susceptible for proteolytic cleavage by Factor Xa, remains unclear.

Factor Va also promotes the binding of Factor Xa to membranes. It forms a stoichiometric (1:1) complex with Factor Xa (27,28,35) with a high affinity for phospholipid surfaces. Thus Factor Va enhances the amount of Xa molecules bound at the membrane and participating in prothrombin activation.

Finally, Factor Va effectuates a shift in the pathway of thrombin formation (Fig. 3). In the absence of Factor Va, large amounts of prethrombin 2 are generated, which are only slowly converted into thrombin (26,36). In the presence of Factor Va, however, there is no detectable prethrombin 2 formation, but substantial amounts of meizothrombin appear as an intermediate product, which are rapidly turned over to thrombin (25).

Two mechanisms have been suggested to explain the effect of Factor Va on the Km for prothrombin activation. The observation that Factor Va induces

clustering of anionic phospholipids (37) led to the proposal that this process contributes to the assembly of the prothrombinase complex. Whether this phenomenon results in the enhancement of the binding affinity of prothrombin for membranes remains to be established.

A direct interaction between Factor Va and prothrombin through its fragment 2 region (21) has also been proposed to account for the Va induced increase of prothrombin-prothrombinase binding affinity. Support for this possibility came from studies which showed that Factor Va enhanced the binding of prothrombin to phospholipid membranes (38). Nevertheless, quantitative data are lacking to relate the effect of Factor Va on the Km to its effect on the prothrombin binding to phospholipids.

Prothrombin activation on cellular surfaces. Coagulation factor activation in vivo is thought to proceed on membranes provided by cells in contact with the blood, such as platelets, endothelial cells, lymphocytes and monocytes. Most extensively studied are blood platelets which are reported to promote both prothrombin as well as Factor X activation (39,40). In vitro, intrinsic factor X activation proceeds by means of "tenase", a complex which like prothrombinase consists of an enzyme (Factor IXa), a protein cofactor (Factor VIIIa) and a phospholipid surface (2,41,42).

It has been suggested that anionic platelet phospholipids account for the procoagulant activity of platelets (43,44,45). Unstimulated- and thrombin stimulated platelets have a low procoagulant activity (46). When platelets are stimulated with collagen a 3- to 7-fold increase of the procoagulant activity is observed, while stimulation by the combined action of thrombin and collagen gives a 10- to 20-fold increase of the platelet procoagulant activity (46). Concomitant with an increase of procoagulant activity is an increase of the amount of exposed phosphatidylserine molecules, the major anionic phosholipid in the platelet plasma membrane (44,45). In resting and thrombin stimulated platelets, phosphatidylserines are located almost exclusively in the cytoplasmic leaflet of the plasma membrane, but upon platelet activation with thrombin plus collagen they are translocated by an as yet unknown mechanism to the outside half of the membrane, where they become in contact with the blood. It has therefore been suggested that phosphatidylserine has an important function in the platelet procoagulant

sites that serve Factor X and prothrombin activation.

Further evidence for this hypothesis came from studies on a patient with a bleeding disorder. It was shown that the platelets of this patient had an impaired Factor X- and prothrombin-converting activity, which coincided with the lack of ability to expose phosphatidylserines at the outside of the platelet plasma membrane (47).

Other investigators have proposed specific receptor proteins as binding sites for the coagulation factors. Such receptors would promote the assembly of prothrombinase and tenase complexes on cellular surfaces. A Factor Xa-specific receptor on the platelet surface was reported (48,49), which was later identified to be platelet-bound factor Va (50,51). Studies of Factor Va-platelet interactions resulted in the postulation of a receptor protein for Factor Va (52,53,54), but its identification was up to now not possible.

Recently, high affinity binding sites for Factor IX and Factor IXa on the endothelial cell were described, that function in the Factor IXa-VIIIa mediated activation of Factor X (55). This endothelial cell surface receptor was identified to consist at least in part of a membrane protein (56). Such receptor proteins for Factor IX/IXa could, however, not be identified on blood platelets (57). Therefore, the precise chemical nature of the procoagulant sites on blood cells is still a matter of debate. The two concepts do, however, not exclude each other and both protein and lipid components may be involved in the binding of coagulation factors.

The present investigation. In this thesis studies on the molecular interactions contributing to the action of prothrombinase on prothrombin are described. Prothrombin activation is studied on phospholipid vesicles as well as on platelet membranes.

During the catalytic conversion of prothrombin, the activation peptides fragment 1 and fragment 1.2 are released. Since both fragments contain the membrane binding domain of prothrombin, while fragment 1.2 has additional affinity for Factor Va via its fragment 2 region, these activation peptides may interfere with the interactions of prothrombin with the prothrombinase complex and thus inhibit prothrombin activation. Chapter II describes the effects of fragment 1 and fragment 1.2 on prothrombin activation by Factor Xa in the presence of various lipid mixtures either with or without Factor

Va. The results presented in this chapter have implications for the mechanisms by which phospholipids and Factor Va affect prothrombin activation.

A somewhat different approach for deriving information about the mode of action of the accessory components in thrombin formation, was found in studies of prothrombin activation by the prothrombin activator from the venom of Oxyuranus scutellatus. This snake venom activator differs in detail from Factor Xa (58,59) and hence may add to our knowledge of the interactions that are important in prothrombin conversion. Chapter III describes the purification, the subunit structure and the catalytic properties of this venom enzyme. The venom activator is used as a tool in studying the platelet procoagulant sites (Chapter IV). Platelets are stimulated with various triggers and their activity in prothrombin activation by Factor Xa is compared with their effect on the venom-catalyzed prothrombin conversion. Also, the number of procoagulant sites for Factor Xa and for the venom enzyme on the platelet surface is determined while information on their chemical nature is inferred from experiments with phospholipase A_2.

Not much is known about the chemical and physical interactions important for membrane binding of the coagulation factors. In early experiments it was shown that lipid surfaces need to possess a net negative charge in order to have a clot promoting activity (60). Furthermore, the activity of phospholipid surfaces depended on their electrostatic potential provided by anionic phospholipids while the chemical nature of the anionic phospholipids would be of minor importance (61). More recent experiments, however, demonstrate that, as far as prothrombinase is concerned, there is no clear relation between the surface charge and the activity of phospholipid vesicles (33,34). Moreover, the kinetic parameters of prothrombin conversion depend on the type of anionic phospholipid that is present in the procoagulant membrane (33).

In Chapter V experiments are outlined in which the surface charge of vesicles and the kind of anionic phospholipid is varied, and their activity in prothrombin conversion is compared. The results favour a chelation model for the calcium-dependent interactions of vitamin K-dependent coagulation factors with phosphatidylserine molecules in the membrane.

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CHAPTER II

THE EFFECTS OF BOVINE PROTHROMBIN FRAGMENT 1 AND FRAGMENT 1.2 ON PROTHROMBIN ACTIVATION

SUMMARY

In this paper we describe the effects of the activation peptides prothrombin fragment 1 and fragment 1.2 on Factor Xa-catalyzed prothrombin activation. Prothrombin activation in free solution by either Factor Xa or Factor Xa together with Factor Va is unaffected by the activation fragments. When negatively charged phospholipids are present we observed considerable inhibition of prothrombin activation by both fragment 1 and fragment 1.2. For the activation of 0.25 μM prothrombin by Factor Xa in the presence of 50 μM phospholipid (phosphatidylserine/phosphatidylcholine, 25/75; mole/mole) and 5 mM $CaCl_2$, 50% inhibition was obtained at 0.28 μM fragment 1 or fragment 1.2. Much higher fragment concentrations were required for 50% inhibition of a prothrombinase complex consisting of Factor Xa, Factor Va, Ca^{2+} and phospholipid. This shows that Factor Va protects prothrombin activation against inhibition by its own activation peptides. Less inhibition by activation fragments was also observed at higher phospholipid and prothrombin concentrations or when the mole fraction phosphatidylserine in the phospholipid vesicles was decreased. The effects of fragment 1 and fragment 1.2 on prothrombin activation were identical throughout all experiments, indicating that the inhibition is due to the γ-carboxyglutamic acid containing region of the activation peptides. Our observations suggest that the activation fragments inhibit prothrombin activation by competing with prothrombin and Factor Xa for binding sites at the phospholipid surface. In such a model Factor Va will protect against the inhibition, since it is known to promote the assembly of the prothrombinase complex through interactions with Factor Xa and prothrombin that are independent of the Gla residues. The kinetic properties of fragment inhibition also suggest that in vivo prothrombin activation will not be affected by the generation of activation peptides.

INTRODUCTION

The activation of the zymogen prothrombin into thrombin is one of the central reactions in blood coagulation. In vivo prothrombin activation is catalyzed by prothrombinase, a complex which consists of the serine protease Factor Xa and the non-enzymatic cofactors phospholipid, Ca^{2+} and Factor Va (1). Factor Xa is capable of hydrolyzing two peptide bonds in the prothrombin molecule. The cleavage of the Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} bonds in bovine prothrombin by Factor Xa leads to the formation of thrombin and the activation peptide fragment 1.2 (2). The reaction product thrombin can cleave the Arg_{156}-Ser_{157} bond of both prothrombin and fragment 1.2, giving rise to fragment 1 and prethrombin 1 or fragment 2, respectively (2,3). In recent years, much has been learned about the function of the non-enzymatic cofactors in Factor Xa-catalyzed prothrombin activation. The stimulatory effect of phospholipids is due to a lowering of the Km for prothrombin (4), whereas the role of Factor Va in prothrombin activation appears to be threefold: a) the Vmax of prothrombin activation is increased 1000-fold by Factor Va (4,5) b) the binding of Factor Xa to phospholipids is promoted by Factor Va (5,6) and c) Factor Va lowers the Km for prothrombin for membranes which have a low affinity for prothrombin (7,8).

Prothrombin and Factor Xa are vitamin K-dependent proteins, containing γ-carboxyglutamic acid residues (9), which are involved in the Ca^{2+}-mediated binding of these proteins to negatively charged phospholipid surfaces. All γ-carboxyglutamic acid residues of prothrombin reside in its fragment 1 region. Since both fragment 1 and fragment 1.2 may be formed during in vivo prothrombin activation, it is possible that the activation peptides can interfere with the interactions of prothrombin with the prothrombinase complex, and hence inhibit prothrombin activation. Indeed it has been stated in literature that fragment 1 can inhibit the generation of thrombin from prothrombin in vivo (10-12).

In this article we present a kinetic study of the effects of fragment 1 and fragment 1.2 on prothrombin activation. Such a study not only provides insight in a possible regulatory role of the activation peptides during in vivo prothrombin activation, but may also give mechanistic information regarding the modes of action of phospholipids and Factor Va in prothrombin activation. The precise mechanisms by which phospholipids (4,7,8) and

Factor Va (7,8) decrease the Km for prothrombin are not yet known. Since fragment 1 can compete with prothrombin for binding to phospholipid vesicles and fragment 1.2 has the additional ability to interact with Factor Va through its fragment 2 region (12), a comparison of the effects of these fragments on prothrombin activation by Factor Xa, in the presence of various kinds of phospholipids, with and without Factor Va, may indicate which interactions are important for the lowering of the Km for prothrombin.

MATERIALS AND METHODS

Materials. S2238, S2337 and I2581 were purchased from AB Kabi Diagnostica, Stockholm. Phe-Pro-Arg-CH_2Cl and dansyl-Glu-Glu-Arg-CH_2CL were obtained from Calbiochem. p-NPGB was from ICN Nutritional Biochemicals. Ovalbumin, HSA (human serum albumin, fatty acid free), STI (soybean trypsin inhibitor), RVV (Russell's viper venom), Echis carinatus venom and PC (1,2-dioleoyl-sn-glycero-3-phosphocholine) were obtained from Sigma. DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-75, Sephadex G-100, Sephacryl S-300 and CNBr-activated Sepharose 4B were supplied by Pharmacia. STI, Factor X, and antibovine Factor X were coupled to CNBr-activated Sepharose 4B according to the method of Cuatrecasas (13).

Phospholipids and phospholipid vesicle preparations. PS (1,2-dioleoyl-sn-glycero-3-phosphoserine) was prepared from PC by enzymatic synthesis as described by Comfurius and Zwaal (14). Single bilayer phospholipid vesicles were obtained by sonication according to the method of de Kruijff et al. (15). Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (16).

Proteins. Bovine prothrombin was purified according to the method of Owen et al (17). Thrombin was purified as described earlier (4). Factor X (18), Factor Xa (19), RVV-X (20) and Factor IXa (21) were purified according to established procedures. Factor V and Factor Va were obtained as described by Lindhout et al (6). Factor VIII:c was purified by a modification of the method of Vehar and Davie (22), as published elsewhere (23). Factor VIII:c

was activated with thrombin as described before (21). Prothrombin fragment 1, fragment 2, and fragment 1.2 were purified as prothrombin activation products (cf.ref. 17) from a reaction mixture containing prothrombin (3 mg/ml) and Factor Xa (50 μg/ml) in 20 mM Tris (pH 7.5), 100 mM NaCl, and 5 mM $CaCl_2$ at 37 °C. For the preparation of fragment 1.2 the prothrombin activation mixture contained 200 μM I2581, a reversible thrombin inhibitor, that prevents feedback reactions of thrombin on prothrombin and fragment 1.2. After 90 min STI-Sepharose (0.25 ml per ml reaction mixture) was added to bind Factor Xa and 10 min later the STI-Sepharose was spun down. In order to irreversibly inhibit thrombin and the remaining Factor Xa, Phe-Pro-Arg-CH_2Cl and dansyl-Glu-Gly-Arg-CH_2Cl were added to the supernatant (final concentrations 16 μM and 1.5 μM, respectively). This mixture was applied to a QAE-Sephadex A-50 column (1.5x30 cm) and the prothrombin activation products were eluted with a linear gradient (2x200 ml) of 0.1 to 0.6 M NaCl in 50 mM Tris (pH 7.5) and 5 mM EDTA. Each activation fragment was subsequently subjected to gel-permeation chromatography on Sephadex G-75 in 50 mM Tris (pH 7.5) and 500 mM NaCl. The activation fragment preparations were finally passed over an anti-bovine Factor X Sepharose 4B column to remove traces of Factor Xa that still contaminated the fragment preparations.

Protein concentrations. The concentrations of thrombin, Factor Xa and Factor IXa were determined by active site titration with p-NPGB (24-26). Prothrombin and Factor X were completely activated with Echis carinatus venom and RVV-X respectively, and their concentrations were subsequently determined by active site titration with p-NPGB. The concentration of Factor Va was determined by kinetic analysis (6) and Factor VIII:c was quantitated as described before (27). The molar concentrations of fragment 1 and fragment 1.2 were calculated from the E_{280} using an $E^{1\%}_{280}$ of 10.1 and a molecular weight of 22,500 for fragment 1 (28) and an $E^{1\%}_{280}$ of 12.3 and a molecular weight of 35,000 for fragment 1.2 (28).

Assay system for measurement of rates of prothrombin activation. Factor Xa and phospholipids either in the absence or presence of fragment 1, fragment 1.2, and/or Factor Va were incubated for 5 min at 37 °C in a buffer containing 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, and 0.5

mg/ml HSA. Prothrombin activation was started by adding prewarmed prothrombin in the same buffer. The final protein concentrations in the reaction mixtures are indicated in the legends to the figures. Rates of prothrombin activation were determined with S2238 as described before (4).

Assay system for the measurement of rates of Factor X activation. Factor IXa and phospholipids, either in the absence or presence of fragment 1, were incubated for 5 min at 37 °C in a buffer containing 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, and 0.5 mg/ml HSA. Factor X activation was started by simultaneous addition of Factor VIIIa (if present) and Factor X, prewarmed in the same buffer. The final protein concentrations in the reaction mixtures are indicated in the legends to the figures. Rates of Factor X activation were determined with S2337 as described before (21).

RESULTS

Effect of fragment 1 and fragment 1.2 on prothrombin activation in the absence of phospholipids. We first investigated whether the activation peptides fragment 1 and fragment 1.2 inhibit Factor Xa-catalyzed prothrombin activation in free solution. In an experiment, in which 1.2 μM prothrombin was activated by either 50 nM Factor Xa or 0.2 nM Factor Xa in the presence of 30 nM Factor Va, we did not detect any inhibition of prothrombin activation at concentrations of fragment 1 or fragment 1.2 between 0 and 10 μM. The conversion of the chromogenic substrate S2337 by Factor Xa was also not affected by fragment 1 and fragment 1.2 concentrations up to 10 μM. These findings indicate that the prothrombin activation fragments do not interact with prothrombin or Factor Xa in a manner by which they interfere with the catalytic activity of Factor Xa on prothrombin per sé.

Effect of fragment 1 and fragment 1.2 on prothrombin activation in the presence of negatively charged phospholipids. In contrast to the reaction in free solution, phospholipid-dependent prothrombin activation was inhibited by the activation fragments (Fig. 1). The inhibition patterns differed considerably depending on whether Factor Va was present or not.

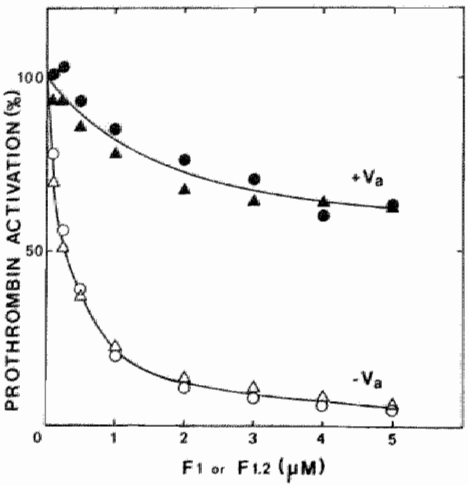

Fig. 1. Effect of fragment 1 and fragment 1.2 on prothrombin activation in the presence of phospholipids. Prothrombin activation was measured in reaction buffer containing 50 μM phospholipid vesicles (PS/PC, 25/75; mole/mole), 0.25 μM prothrombin and 2 nM Factor Xa (open symbols) or 1 pM Factor Xa and 5 nM Factor Va (closed symbols). The concentration of fragment 1 (○-○,●-●) or fragment 1.2 (△-△,▲-▲) present are indicated in the figure. Rates of prothrombin activation are expressed relative to the rate measured in the absence of activation fragments which was taken as 100%. Without Factor Va 100% = 2.07 moles prothrombin activated/min/mol Xa and with Factor Va 100% = 3093 moles prothrombin activated/min/mol Xa.

The activation of 0.25 μM prothrombin by Factor Xa in the presence of 50 μM phospholipid vesicles was strongly inhibited by both fragment 1 and fragment 1.2. The inhibition characteristics of fragment 1 and fragment 1.2 did not differ and both activation fragments caused 50% inhibition at a concentration of 0.28 μM. Much less inhibition was observed when saturating amounts of Factor Va are included in the reaction mixtures. Although both activation fragments gave the same inhibition pattern, fragment concentrations exceeding 5 μM had to be added to obtain 50% inhibition. The protective effect of Factor Va was not only found in this experiment but is a general feature observed in all experiments in which Factor Va was present.

Fragment 2, the prothrombin activation peptide which lacks Gla residues, but which has retained the ability to interact with Factor Va (12), did not inhibit prothrombin activation, irrespective of whether phospholipid and/or Factor Va were present or not. In the present study this activation peptide was therefore not further examined.

Effect of the phospholipid concentration on the inhibition of prothrombin activation by fragment 1 and fragment 1.2. Since the experiments presented in the previous paragraphs indicate that inhibition of prothrombin activation by fragment 1 and fragment 1.2 only occurs in the presence of phospholipid, we decided to investigate whether the extent of inhibition

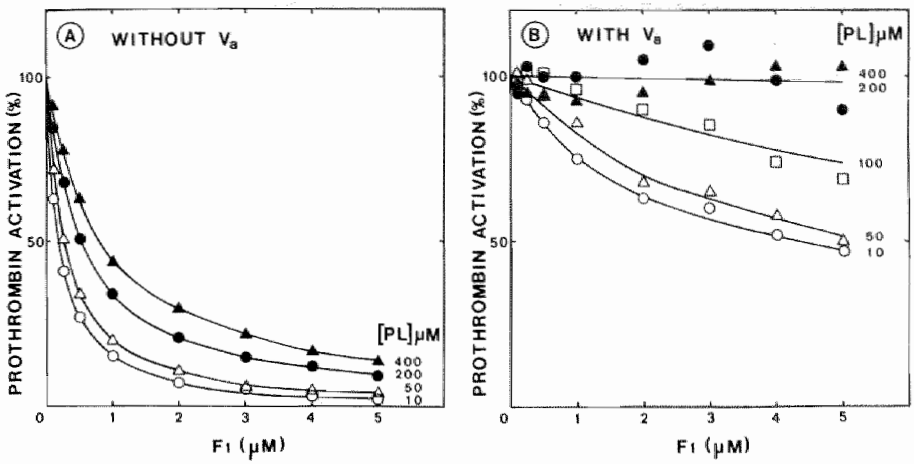

Fig. 2. **Inhibition of prothrombin activation by fragment 1 at different phospholipid concentrations.** Prothrombin activation was measured in reaction buffer containing 0.25 μM prothrombin, amounts of fragment 1 indicated in the figure and 2 nM Factor Xa (Fig. 2A) or 1 pM Factor Xa and 5 nM Factor Va (Fig. 2B). The amounts of phospholipid vesicles (PS/PC, 25/75; mole/mole) present were 10 μM (○-○), 50 μM (△-△), 100 μM (□-□), 200 μM (●-●), or 400 μM (▲-▲). Rates of prothrombin activation are expressed relative to the rate measured in the absence of fragment 1, which was taken as 100%. Without Factor Va (A) 100% was 1.15 (○-○), 1.67 (△-△), 0.93 (●-●), or 0.64 (▲-▲) moles prothrombin activated/min/mol Xa. With Factor Va (B) 100% was 2332 (○-○), 2664 (△-△), 1314 (□-□), 809 (●-●), or 522 (▲-▲) moles prothrombin activated/min/mol Xa.

depends on the amount of phospholipid present in the reaction mixture. In Fig. 2 it is shown that inhibition of prothrombin activation by fragment 1 was less pronounced at higher phospholipid concentrations. This phenomenon was observed both in the absence (Fig. 2A) and presence of Factor Va (Fig. 2B). For the complete prothrombin activating complex (Factor Xa, Va, Ca^{2+} and phospholipid) it was even impossible to detect any inhibition by fragment 1 at phospholipid concentrations of 200 uM or higher. Fragment 1.2 showed the same inhibition characteristics (data not shown) and could not be distinguished from fragment 1.

Inhibition of prothrombin activation by fragment 1 and fragment 1.2 in the presence of phospholipid vesicles containing various mol % PS. The phospholipid vesicles used in the previous experiments contained 25 mol % PS. These vesicles are reported to have a high affinity for prothrombin and

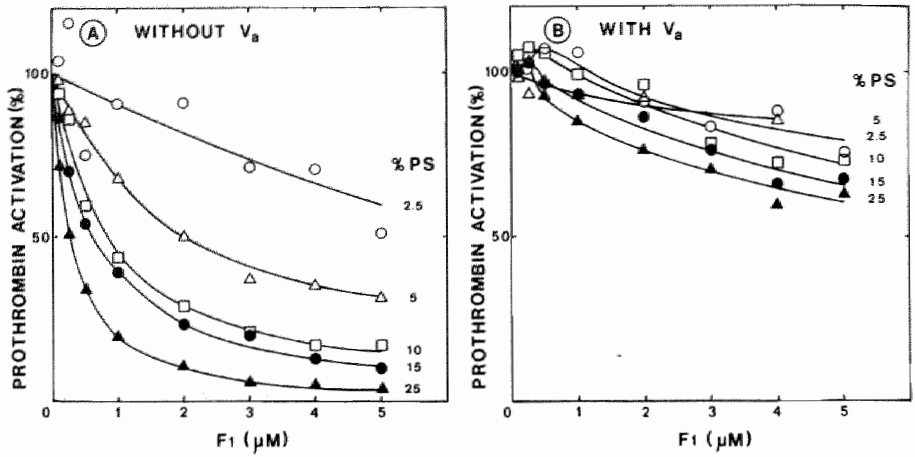

Fig. 3. **Inhibition of prothrombin activation by fragment 1 measured in the presence of phospholipid vesicles with varying mol % PS.** Prothrombin activation was measured in reaction buffer containing 50 μM phospholipid, 0.25 μM prothrombin, amounts of fragment 1 indicated in the figure, and 2 nM Factor Xa (Fig. 3A), or 1 pM Factor Xa and 5 nM Factor Va (Fig. 3B). The phospholipid vesicles contained the following mol % PS (supplemented to 100% with PC): 2.5% PS (○-○) 5% PS (△-△), 10% PS (□-□), 15% PS (●-●), 25% PS (▲-▲). The rates of prothrombin activation are expressed relative to the rate measured in the absence of fragment 1 which was taken as 100%. Without Factor Va (A) 100% was 0.0084 (○-○), 0.047 (△-△), 0.227 (□-□), 0.511 (●-●) or 1.98 (▲-▲) moles prothrombin activated/min/mol Xa. With Factor Va (B) 100% was 1840 (○-○), 3360 (△-△), 2796 (□-□), 2971 (●-●) or 3170 (▲-▲) moles prothrombin activated/min/mol Xa.

Factor Xa (29,30). Although no binding affinities are reported for fragment 1 or fragment 1.2 it is likely that they are similar to those for prothrombin. The affinity of vitamin K-dependent coagulation factors for phospholipid bilayers decreases when the mol fraction of negatively charged phospholipid is decreased (29,30). Fig. 3 shows the effect of variation of the mol % PS in phospholipid vesicles on the inhibition of prothrombin activation by fragment 1. The most striking effect was observed again in the absence of Factor Va (Fig. 3A). Fragment 1 was an excellent inhibitor of prothrombin activation at vesicles containing a high mol % PS. When the mol % PS in the phospholipid vesicles was decreased from 25 to 2.5 %, there was a considerable decrease of the potency of fragment 1 to inhibit prothrombin activation. Fifty percent inhibition was observed at 0.28 μM fragment 1 for vesicles containing 25 mol % PS whereas for vesicles con-

taining 2.5 mol % PS a fragment 1 concentration of about 5 μM was required to obtain the same level of inhibition. It should be emphasized that the rates of prothrombin activation for each individual kind of phospholipid are expressed relative to that measured in the absence of fragment 1. Of course the rates of prothrombin activation at low mol % PS were appreciably lower than those determined at high mol % PS (cf. ref. 8).

In the presence of Factor Va the effect of variation of the mol % PS on the inhibition of prothrombin by fragment 1 was much less pronounced (Fig. 3B). Prothrombin activation by Factor Xa-Va in the presence of vesicles with a high PS content was already poorly inhibited and a decrease of the amount of PS in the phospholipid membrane caused a further decrease of inhibitory effect of fragment 1. In some cases we even observed some stimulation of prothrombin activation at low fragment 1 concentrations. In the discussion we will give a possible explanation for this stimulatory effect. The experiment presented in Fig. 3 was repeated with fragment 1.2. This activation peptide showed the same inhibition pattern as was observed for fragment 1 (data not shown).

Effect of fragment 1 and fragment 1.2 on prothrombin activation at different prothrombin concentrations. The kinetic properties of the prothrombinase complex are satisfactorily explained in a model in which phospholipid-bound prothrombin is activated by phospholipid-bound Factor Xa (4,8,31,32). In this paper we observe that inhibition of prothrombin activation by fragment 1 and fragment 1.2 only occurs in the presence of phospholipid and that the inhibition is less at higher phospholipid concentrations and at vesicles with a low binding affinity for the activation fragments. Therefore, it is likely that fragment 1 and fragment 1.2 interfere with prothrombin activation via interaction with the phospholipid surface. Binding of fragment 1 and fragment 1.2 to phospholipid will lower the number of surface-binding sites for prothrombin and/or Factor Xa which will result in an inhibition of prothrombin activation. The inhibition will than have a competitive character, since it originates through competition of the activation fragments with prothrombin and/or Factor Xa for binding sites at the phospholipid surface. In that case, inhibition by activation fragments will be less at higher prothrombin (substrate) concentrations. Indeed it was found that increased prothrombin concentrations protect

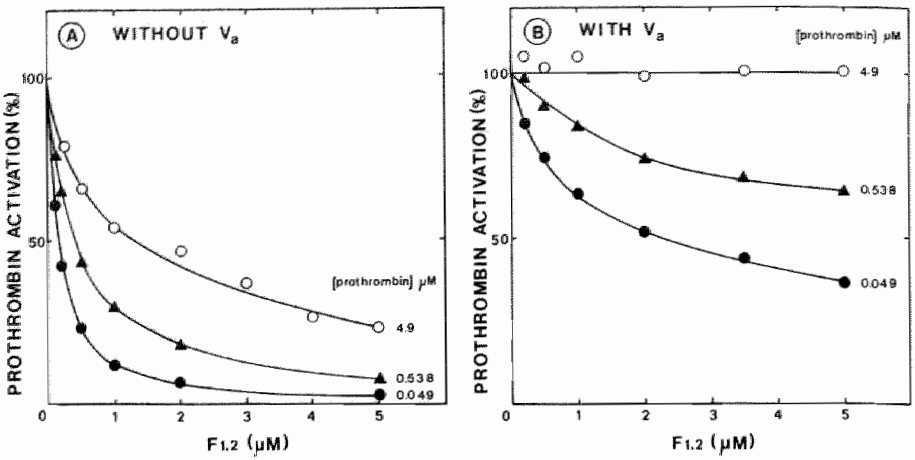

Fig. 4. **Inhibition of prothrombin activation by fragment 1.2 measured at different prothrombin concentrations.** Prothrombin activation was measured in reaction buffer containing 50 μM phospholipid, (PS/PC, 25/75; mole/mole), amounts of fragment 1.2 indicated in the figure and 2 nM Factor Xa (Fig. 4A), or 1 pM Factor Xa and 5 nM Factor Va (Fig. 4B). The reaction mixtures further contained 0.049 μM (●-●), 0.538 μM (▲-▲), or 4.9 μM (○-○) prothrombin. The rates of prothrombin activation are expressed relative to the rate measured in the absence of fragment 1.2 which was taken as 100%. Without Factor Va (A) 100% was 0.68 (●-●), 1.98 (▲-▲), or 2.46 (○-○) moles prothrombin activated/min/mol Xa. With Factor Va (B) 100% was 1111 (●-●), 3052 (▲-▲), or 3081 (○-○) moles prothrombin activated/min/mol Xa.

against the inhibition by fragment 1.2 (Fig. 4). In the absence of Factor Va, a 10-fold higher fragment 1.2 concentration was required to obtain 50% inhibition when the prothrombin concentration was raised from 0.049 μM to 4.9 μM (Fig. 4A). A similar protective effect of prothrombin was observed in the presence of Factor Va (Fig. 4B). Under these conditions the inhibition of prothrombin activation by fragment 1.2 could even be completely abolished at high prothrombin concentrations.

Kinetic characterization of the type of inhibition of prothrombin activation by fragment 1 and fragment 1.2. Further insight in the type of inhibition of enzymatic reactions can be obtained from Lineweaver-Burk plots made at various concentrations of inhibitor. Fig. 5 shows the result of such an approach for the inhibition of prothrombin activation by fragment 1. In the absence of Factor Va, Lineweaver-Burk plots were made at

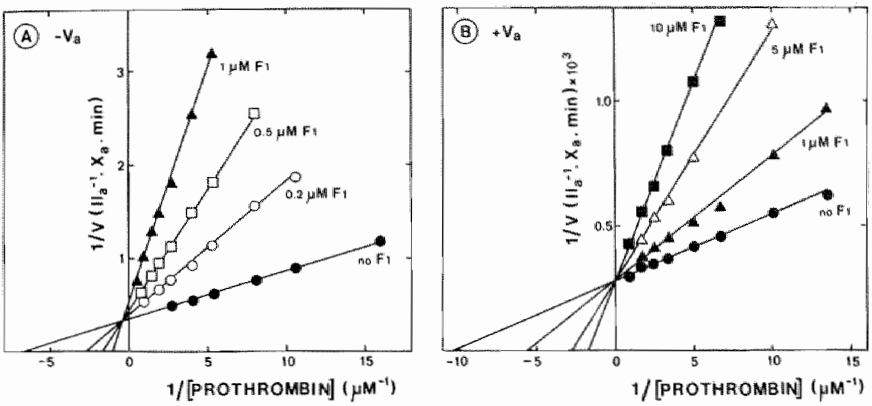

Fig. 5. **Lineweaver-Burk plots of prothrombin activation measured in the presence of different concentrations fragment 1.** Prothrombin activation was measured in reaction buffer containing 50 μM phospholipid (PS/PC, 25/75; mole/mole), varying concentrations prothrombin, 0 μM (●-●), 0.2 μM (○-○), 0.5 μM (□-□), 1 μM (▲-▲), or 5 μM (△-△) fragment 1, and 2 nM Factor Xa (Fig. 5A), or 1 pM Factor Xa and 5 nM Factor Va (Fig. 5B). Lineweaver-Burk plots were drawn after statistical analysis as described by Eisenthal and Cornish-Bowden (33).

0, 0.2, 0.5 and 1.0 μM fragment 1 (Fig. 5A). It is obvious that the inhibition was not of a single type since upon variation of the fragment 1 concentration both the Km and the Vmax changed. It is possible that the increase of the Km is caused by a competition of fragment 1 with prothrombin whereas the decrease of the Vmax at higher fragment concentrations reflects competition with Factor Xa for binding sites at the phospholipid surface. In the presence of Factor Va higher concentrations of fragment 1 were required to obtain an effect on the Km for prothrombin (Fig. 5B). This is presumably caused by the fact that Factor Va protects the prothrombinase complex against inhibition by the activation fragments. Factor Va also abolished the effect of activation fragments on the Vmax. This supports the concept that the decrease of the Vmax by activation fragments is due to competition with Factor Xa for surface-binding sites, since it is well established that Factor Va promotes the binding of Factor Xa to negatively charged phospholipids (5,6). It should be mentioned, however, that in the presence of Factor Va and fragment 1 an upward curvature of Lineweaver-Burk plots was observed at low prothrombin concentrations (< 0.06 μM, data not

shown). This indicates that care should be taken in interpretation of the kinetic data. In the absence of Factor Va, Lineweaver-Burk plots were straight lines over a wide range of prothrombin concentrations (0.03-2 μM).

Effect of fragment 1 on intrinsic Factor X activation. When the inhibition of prothrombin activation by the Gla-containing activation fragments 1 and 1.2 is due to a competition with prothrombin and/or Factor Xa for binding sites at the phospholipid surface, one would expect that both activation fragments would also inhibit intrinsic Factor X activation.

Like prothrombin activation this reaction also takes place at a negatively charged phospholipid surface (21). Indeed, we observed that Factor X activation by Factor IXa in the presence of phospholipid could be inhibited by fragment 1 (Fig. 6), although a high concentration (5 μM) was required to obtain 50% inhibition. Factor VIIIa protects against the inhibition by fragment 1. In the presence of this cofactor there was hardly any inhibition. At low fragment 1 concentrations there was even a stimulation of Factor X activation.

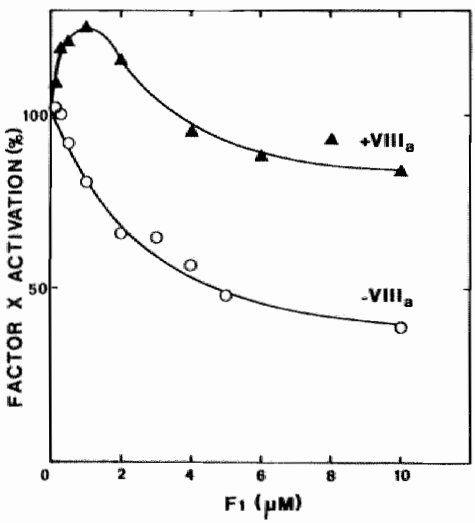

Fig. 6. Inhibition of intrinsic Factor X activation by fragment 1. Intrinsic Factor X activation was measured in reaction buffer containing 50 μM phospholipid (PS/PC, 25/75; mole/mole), 0.25 μM Factor X, amounts of fragment 1 indicated in the figure, and 96 nM Factor IXa (○-○), or 25 nM Factor IXa and 0.02 nM Factor VIIIa (▲-▲). Rates of Factor X activation are expressed relative to the rate measured in the absence of fragment 1 which is taken as 100%. Without Factor VIIIa 100% is 0.444 nM Xa formed/min and with Factor VIIIa 100% is 5.25 nM Xa formed/min.

DISCUSSION

The data presented in this article demonstrate that the Gla-containing activation peptides prothrombin fragment 1 and prothrombin fragment 1.2 can

inhibit Factor Xa-catalyzed prothrombin activation in vitro. Since inhibition only occurs when negatively charged phospholipids are part of the prothrombin-activating complex, it is likely that the activation fragments interfere with prothrombin activation through an interaction with the phospholipid surface. It is now well established that prothrombin activation in the presence of phospholipids is a surface process in which phospholipid-bound prothrombin is activated by phospholipid-bound Factor Xa (4,7,8,30,31). The precise mode of action of phospholipids differs, however, dependent on whether Factor Va is present or not (7,8). In the absence of Factor Va, the prothrombin that is converted by phospholipid-bound Factor Xa can originate from a dense shell of bound prothrombin that surrounds the phospholipid membrane (4,8,32), whereas in the presence of Factor Va the formation of the prothrombin-prothrombinase complex is the result of multiple interactions of soluble prothrombin with Factor Xa, Factor Va and phospholipids (7,8,31). In both situations occupation of binding sites at the phospholipid surface by activation fragments will interfere with the interaction of prothrombin and Factor Xa with phospholipid resulting in a decrease of the rate of prothrombin activation. The possibility for different modes of action of phospholipid in the absence and presence of Factor Va is also supported by the experiments presented in this paper. In the absence of Factor Va both fragment 1 and fragment 1.2 are excellent inhibitors of prothrombin activation, whereas much less inhibition is observed in the presence of Factor Va.

The strong inhibition by activation fragments in the absence of Factor Va is explained in a model in which the fragments compete with prothrombin and Factor Xa for binding sites at the phospholipid surface. Competition with prothrombin will lower the effective substrate concentration available for reaction with Factor Xa and competition with Factor Xa will lower the amount of phospholipid-bound enzyme that participates in prothrombin activation. The inhibition has competitive characteristics since less inhibition is observed at higher prothrombin concentrations. A reduction of the extent of inhibition was also found at higher phospholipid concentrations, or when phospholipids with lower PS content were utilized in the prothrombinase complex. In a "competitive model" more activation fragments will be required at higher phospholipid concentrations, since a larger number of binding sites has to be occupied, in order to effectively reduce

the concentrations of phospholipid-bound prothrombin and Factor Xa. At phospholipid vesicles with a low mol percentage PS, efficient occupation of binding sites will also require more activation fragment since such vesicles are known to have a low affinity for vitamin K-dependent coagulation factors (29,30).

The type of inhibition of prothrombin activation by fragment 1 and fragment 1.2 is not purely competitive, since Lineweaver-Burk plots show that upon an increase of the activation fragment concentration the Km for prothrombin increases and the Vmax of prothrombin activation decreases. This is not surprising since pure competitive inhibition requires competition between inhibitor and substrate for binding to the active site of Factor Xa which is apparently not the case in our experiments. We believe that the increase of the Km results from the competition of activation fragments with prothrombin binding to phospholipids and that the decrease of the Vmax is caused by a competition with Factor Xa for phospholipid.

Factor Va protects the prothrombinase complex against inhibition by activation fragments. In the presence of Factor Va, almost no inhibition was observed at high phospholipid or prothrombin concentrations, or when prothrombin activation was measured in the presence of phospholipid vesicles with a low mol % PS. The protective effect of Factor Va may reflect its role in the assembly of the prothrombinase complex. Factor Va is reported to enhance the binding of Factor Xa to negatively charged phospholipids (5,6) and to promote the interaction of prothrombin with the enzymatic unit of the prothrombinase complex (8,31). These interactions are presumably independent of the γ-carboxyglutamic acid residues of prothrombin and hence not liable to interference by activation fragments. Such Gla-independent interactions may also be involved in the assembly of the intrinsic Factor X-activating complex and can provide the explanation for the fact that Factor VIIIa protects this complex against inhibition by prothrombin activation fragments.

In an earlier paper (8), we presented kinetic evidence in support of the proposal of Nelsestuen (31) that the low Km for prothrombin of prothrombinase in the presence of Factor Va is the result of prothrombin-Factor Va and prothrombin-phospholipid interactions that occur near the active site of prothrombinase. Binding of prothrombin to phospholipid outside the

enzymatic domain is actually unfavourable since it lowers the effective prothrombin concentration available for interaction with the enzymatic unit. This may explain why in certain experiments a stimulation of prothrombin activation was observed at low activation fragment concentrations (Fig. 2B and Fig. 3B). The action of the activation fragments can be twofold. They can stimulate prothrombin activation by increasing the effective prothrombin concentration through removal of prothrombin from the excess of phospholipid binding sites, whereas they inhibit prothrombin activation by competition for phospholipids that participate in the interactions at the enzymatic unit. It is possible that under some conditions the stimulatory effect of activation fragments exceeds their inhibitory effect. A similar explanation can be given for the stimulation of intrinsic Factor X activation in the presence of Factor VIIIa (Fig. 6).

The data presented in this paper also contain information regarding the way by which Factor Va increases the affinity of prothrombin for a prothrombinase complex that contains phospholipids with a low affinity for vitamin K-dependent coagulation Factors (7,8). This can be accomplished by a) a direct interaction of prothrombin with Factor Va (8,31), probably through its fragment 2 region, or b) a Factor Va induced clustering of negatively charged phospholipid molecules around the enzymatic unit that creates a better surface for prothrombin binding (8), or c) (not mentioned before) a Factor Va induced increase of a rate constant in the pathway of prothrombin activation that simultaneously increases the k_{cat} and decreases the Km. In a simplified reaction scheme for serine proteases

$$E + S \underset{}{\overset{Ks}{\rightleftharpoons}} ES \underset{P_1}{\overset{k2}{\rightarrow}} EA \xrightarrow{k3} E + A$$

$$k_{cat} = \frac{k2.k3}{k2+k3} \text{ and } Km = Ks. \frac{k3}{k2+k3}$$

In such a mechanism, it is possible that an increase of a single rate constant (k2) by Factor Va can cause an increase of k_{cat} and a decrease of the Km. Using fragment inhibition patterns as a tool to distinguish between these possibilities one would expect that:

1. fragment 1.2 is a better inhibitor than fragment 1 in case of prothrombin activation in the presence of Factor Va and low affinity phospholipids in possibility a
2. fragment 1 and fragment 1.2 are excellent inhibitors of prothrombinase in the presence of Factor Va and low affinity phospholipid in possibility b
3. both fragment 1 and fragment 1.2 are poor inhibitors in possibility c, since Factor Va exerts its effect on the Km for prothrombin via an increase of a rate constant which will likely not be affected by either activation fragment.

The experiments presented in this paper neither support possibility a) nor b) and are on basis of exclusion in favour of possibility c). We are of course aware of the fact that this reasoning is more or less speculative, since it is assumed that the isolated activation fragments have retained full capacity to interact with phospholipids (fragment 1 and fragment 1.2) or Factor Va (fragment 1.2), which may not be the case. It emphasizes however, that possibility c) has to be taken into account in future considerations on the mechanism of action of Factor Va in prothrombin activation.

It has been reported that there is no significant fragment 1 formation during prothrombin activation in blood and plasma (34). The major activation peptide demonstrated in serum was fragment 1.2. It is unlikely that fragment 1.2 plays a regulatory role during in vivo prothrombin activation. In vivo prothrombin activation occurs at approximately 2 μM prothrombin in the presence of Factor Va at a membrane surface that likely contains a low density of negatively charged phospholipids (35). Our data indicate that under these conditions the activation fragments hardly affect prothrombin activation.

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CHAPTER III

PROTHROMBIN ACTIVATION BY AN ACTIVATOR FROM THE VENOM OF OXYURANUS SCUTELLATUS (TAIPAN SNAKE)

SUMMARY

The prothrombin activator from the venom of Oxyuranus scutellatus (Taipan snake) was purified by gel filtration on Sephadex G-200 and ion-exchange chromatography on QAE-Sephadex. The activator is a large protein with a molecular weight of approximately 300,000, which is composed of subunits of M_r 110,000 and 80,000 and two disulfide-linked polypeptides of M_r 30,000. One or both of these M_r 30,000 subunits contain the active site. The venom activator readily converts Factor Xa-specific chromogenic substrates and is also able to activate prothrombin (Km = 166 μM, Vmax = 2.5 μmoles prothrombin activated/min/mg venom). Gel electrophoretic analysis of prothrombin activation indicates that the venom activator randomly cleaves the Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} bonds of prothrombin since both thrombin and meizothrombin are formed as reaction products. Venom-catalyzed prothrombin activation is not affected by bovine Factor Va but is greatly stimulated by phospholipids plus Ca^{2+} ions. This stimulatory effect is explained by a decrease of the Km for prothrombin. In the presence of 50 μM phospholipid vesicles (phosphatidylserine/phosphatidylcholine, 25/75; mole/mole) the Km is 0.34 μM and the Vmax is 7.1 μmoles prothrombin activated/min/mg venom. The purified venom activator contains γ-carboxyglutamic acid residues which presumably function in the interaction between the venom activator and phospholipids. Treatment of the activator with 0.8 M NaSCN strongly reduces its ability to activate prothrombin but has no effect on its amidolytic activity. The prothrombin-converting activity of the NaSCN-treated activator can be restored with bovine Factor Va.

During prolonged gradient gel electrophoresis the M_r 300,000 activator dissociates into smaller subunits. This causes a loss of the prothrombin-converting activity, while the amidolytic activity is recovered in a protein with an apparent molecular weight of 57,000. This protein can,

however, rapidly activate prothrombin in the presence of Factor Va or in the presence of a protein component of M_r 220,000 that also migrates in the gel. These results suggest that the prothrombin activator from the Oxyuranus scutellatus venom is a multimeric protein complex consisting of a Factor Xa-like enzyme and a Factor Va-like cofactor.

INTRODUCTION

A central reaction in blood coagulation is the activation of the zymogen prothrombin to the serine protease thrombin. This reaction, which is catalyzed by Factor Xa, is accelerated by Ca^{2+} ions, phospholipids and Factor Va. Kinetic studies have shown that phospholipids promote prothrombin activation by decreasing the Km for prothrombin (1), whereas Factor Va enhances prothrombin activation by a) causing a 1000-fold increase in the k_{cat} of prothrombin activation (1,2), b) promoting the binding of Factor Xa to the phosholipid surface (2,3) and c) lowering the Km for prothrombin, a feature which becomes most striking when phospholipids with a low affinity for prothrombin are used (4,5). However, the molecular mechanisms underlying these kinetic effects remain unclear.

Several snake venoms contain procoagulant proteins that can activate zymogens that participate in blood coagulation. Since the mechanisms by which venom enzymes activate coagulation factors often differ from those of the mammalian enzymes, venom activators can provide additional information about the molecular mechanisms of coagulation factor activation. With respect to prothrombin activation, venom activators can have widely different properties. The prothrombin activator from Echis carinatus rapidly converts prothrombin into meizothrombin (6,7) in a reaction that is not affected by phospholipid or Factor Va. Prothrombin activation by the activator from Notechis scutatus scutatus (Mainland Tiger snake) is greatly stimulated by both Factor Va and phospholipids (8,9), whereas prothrombin activation by the venom activator from Oxyuranus scutellatus (Taipan snake) is enhanced by the presence of phospholipids (10,11).

In this paper, we describe the purification and the structural and functional characterization of the prothrombin activator from Oxyuranus scutellatus venom. It is shown that this venom activator is a multimeric

protein complex comprised of a catalytic unit associated with a cofactor part that is required for efficient prothrombin activation. The catalytic unit has many properties in common with Factor Xa, whereas the cofactor part presumably functions like Factor Va.

EXPERIMENTAL PROCEDURES

Reagents. S2238, S2337, S2222 and I2581 were purchased from AB Kabi Diagnostica. Ovalbumin, human serum albumin (fatty acid free), benzamidine-HCl and 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) were from Sigma. Dansyl-Glu-Gly-Arg-CH_2Cl was purchased from Calbiochem and p-NPGB was from ICN Nutritional Biochemicals. Heparin (USP activity 175 U/mg) was obtained from Organon. DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-100 and G-200, polyacrylamide gradient gels PAA 4/30 and calibration kits for electrophoresis were purchased from Pharmacia. Lyophilized crude snake venoms were obtained from Sigma. As indicated by the manufacturer the venom of Oxyuranus scutellatus may be from the subspecie canni (papuan taipan) or scutellatus (australian taipan) or a mixture of both.

Proteins. Bovine prothrombin was purified according to the method of Owen et al. (12). Thrombin was purified as described earlier (1). Bovine Factor Xa was purified by the method of Fujikawa et al. (13). Factor V and Factor Va were obtained as described by Lindhout et al. (3). The protein preparations were stored at -80 °C in 50 mM Tris-HCl, 175 mM NaCl, 0.5 mg/ml human serum albumin (pH 7.9). Factor Va was stored in the same buffer containing 5 mM $CaCl_2$. Purified venom preparations were stored at -80 °C in 50 mM Hepes, 150 mM NaCl, 10 mM benzamidine (pH 7.2).

Protein concentrations. The molar concentrations of thrombin (14) and Factor Xa (15) were determined by active site titration with p-NPGB. Prothrombin concentrations were determined by the same method after complete activation with Echis carinatus venom. Factor Va concentration was determined by kinetic analysis as described by Lindhout et al. (3). The concentration of purified activator from Oxyuranus scutellatus was

calculated from a protein determination according to Sedmak et al. (16), using bovine serum albumin as a standard, and assuming a molecular weight of 300,000 for the venom activator (see results).

Phospholipids and phospholipid vesicle preparations. PS was prepared from PC by enzymatic synthesis (17). Single bilayer phospholipid vesicles were prepared by sonication of mixtures of 25 mol % PS and 75 mol % PC, or 5 mol % PS and 95 mol % PC as described before (1). Phospholipid concentrations were determined by phosphate analysis according to Böttcher et al. (18).

Assay system for measurement of prothrombin activation. The activation of prothrombin by Oxyuranus scutellatus was followed by measuring the generation of amidolytic activity (thrombin plus meizothrombin, cf. ref. 19) towards the chromogenic substrate S2238. Appropriate dilutions of the venom activator were preincubated for 5 minutes at 37 ^{o}C in a buffer containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl and 0.5 mg/ml human serum albumin. When present, the cofactors $CaCl_2$, phospholipid vesicles, or Factor Va were included in the preincubation mixture. Prothrombin activation was started with the addition of prewarmed prothrombin in the same buffer. After different time intervals, samples were taken and transferred to cuvettes containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin and 235 µM S2238 (1 ml final volume). The conversion of S2238 was followed by measuring the absorbance change on an Aminco DW-2C spectrophotometer set in the dual wavelength mode at 405-500 nm. From a calibration curve, made with known amounts of active site-titrated thrombin, the amounts of activated prothrombin in the reaction mixture could be determined.

Determination of the catalytic activity of the venom activator. The prothrombin-converting activity of venom activator preparations was routinely assayed in reaction mixtures that contained 0.5 µM prothrombin, 50 µM phospholipid (PS/PC, 25/75; mole/mole), 5 mM $CaCl_2$ either with or without 5 nM Factor Va and appropriate dilutions of the venom activator preparation. The rate of prothrombin activation was determined as described above.

The amidolytic activity of the venom activator was measured in cuvettes containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml

ovalbumin, and 197 µM S2337. The conversion of S2337 was followed by measuring the absorbance change on an Aminco DW-2C spectrophotometer set in the dual wavelength mode at 405-500 nm. The rate of p-nitroaniline formation was calculated using an E_{405} of 1.04×10^4 $mol^{-1}.l.cm^{-1}$ for p-nitroaniline.

Gel electrophoretic techniques. The subunit structure of the venom prothrombin activator was analyzed by electrophoresis in the presence of SDS on acrylamide tube gels according to the method of Laemmli (20). The molecular weight of the venom activator was determined by electrophoresis under non-denaturing conditions on Pharmacia gradient gels (PAA 4/30) following the instructions of the manufacturer. Analysis of the activation products generated during prothrombin cleavage by the venom activator was carried out by SDS-polyacrylamide gel electrophoresis on 10% acrylamide slab gels according to the method of Laemmli (20). Further experimental details are given in the legends to the figures.

RESULTS

Purification and characterization of the prothrombin activator from Oxyuranus scutellatus. The prothrombin-activating enzyme from Oxyuranus scutellatus was purified from the crude venom by gel filtration on Sephadex G-200 followed by QAE-Sephadex ion-exchange chromatography. In order to prevent autodegradation of the activator, 10 mM benzamidine was present in all buffers used in the purification procedure. Detailed information regarding this purification procedure is given in the legend to Table I. Separation on Sephadex G-200 gave five major protein peaks, the first of which contained both prothrombin-converting and S2337 hydrolase activity. Purification in this step was about 10-fold with a 66% yield (Table I). QAE-Sephadex ion-exchange chromatography gave a further 2-fold purification and a final overall yield of 30%. The gain in specific activity indicates that the activator comprised at most 5% of the crude venom. Walker et al. (11) reported that the crude venom of Oxyuranus scutellatus scutellatus contains an inhibitor of the coagulant and amidolytic activity that was removed during the isolation procedure. The crude venom of Oxyuranus

Table I

Purification of the prothrombin activator from the venom of Oxyuranus scutellatus

Crude venom (100 mg) was dissolved in 4 ml 50 mM Hepes (pH 7.2), 150 mM NaCl, 10 mM benzamidine, and applied to a Sephadex G-200 column (2.5x90 cm) at a flow rate of 15 ml/h at 4 °C. After elution, the fractions containing prothrombin-converting activity were pooled and applied to a QAE-Sephadex A-50 column (1x20 cm). The activator adhered to the resin and was eluted with a linear gradient (2x60 ml) of 150 to 350 mM NaCl in 50 mM Hepes (pH 7.2), 10 mM benzamidine. The activator eluted in a single peak at about 200 mM NaCl. The prothrombin-converting activity of the venom activator was assayed as described in the experimental procedures.

	Total protein (mg)	Specific Activity (nmoles prothrombin activated/min/ug venom)	Recovery (%)	Purification Factor
Crude venom	100	0.2	100	1
Sephadex G-200	6.3	2.1	66	10
QAE Sephadex	1.7	3.5	30	18

scutellatus (subspecies scutellatus or canni or a mixture of both) used as starting material in our study apparently did not contain such an inhibitor since neither the recovery of amidolytic activity nor the recovery of prothrombin-converting activity was increased during the purification procedure.

SDS gel electrophoresis of the purified activator on 7.5% acrylamide tube gels (Fig. 1) showed four major protein bands with apparent molecular weights of 220,000, 120,000, 80,000 and 60,000. Upon reduction with β-mercaptoethanol three bands were present with molecular weights of 110,000, 80,000 and 30,000. The activator could be irreversibly inhibited with the active site-directed chloromethyl ketone dansyl-Glu-Gly-Arg-CH_2Cl. Gels of the dansyl-Glu-Gly-Arg-CH_2Cl-inhibited venom activator showed that the fluorescent inhibitor was almost exclusively incorporated in the M_r 60,000 band on non-reduced gels and in the M_r 30,000 band on reduced gels (Fig. 1). This indicates that the M_r 60,000 subunit contains the active site of the venom activator.

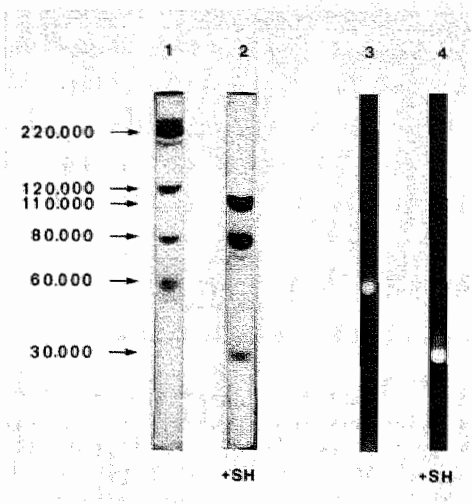

Fig. 1. **Polyacrylamide gel electrophoresis of the purified prothrombin activator from the venom of Oxyuranus scutellatus.** Active site-labeled activator was obtained by incubating 2.5 ml of activator (62 µg/ml) with 40 µM dansyl-Glu-Gly-Arg-CH_2Cl at 22 °C for 20 min. Excess inhibitor was removed by dialysis, and the activator was concentrated in an Amicon B15 concentration cell. 33 µg of activator (lanes 1 and 2) or active site-labeled activator (lanes 3 and 4) were subjected to gel electrophoresis on 7.5% gels in the presence of sodium dodecyl sulfate according to Laemmli (20). 5% β-mercaptoethanol was present in reduced samples. The gels containing activator were stained with Coomassie Brilliant Blue R-250, and the gels containing the active site-labeled activator were photographed under UV light using a yellow filter (cutoff-400 nm). The molecular weights of the subunits, which are indicated in the figure, were determined in a separate experiment on 5% gels (subunits with M_r > 100,000) or 10% gels (subunits with M_r < 100,000).

Catalytic properties of the purified venom activator. The purified prothrombin activator from Oxyuranus scutellatus venom readily cleaves a number of commercially available chromogenic substrates that have been designed for Factor Xa. The kinetic parameters for chromogenic substrate conversion by the venom activator and by bovine Factor Xa, which were obtained from Lineweaver-Burk plots (Fig. 2), are compared in Table II. For all chromogenic substrates tested, the amidolytic activity of the venom activator was lower than that of Factor Xa. Factor Va and Ca^{2+} ions had no effect on the catalytic efficiency.

The purified venom activator was also able to activate prothrombin. Venom-catalyzed prothrombin activation was stimulated about eight times by Ca^{2+} ions and was not affected by the presence of Factor Va. Phospholipids (50 µM) plus Ca^{2+} ions (5 mM) caused a 3000-fold rate enhancement of prothrombin activation by the venom activator. This stimulatory effect of phospholipids plus Ca^{2+} was observed both in the absence and presence of Factor Va. Further details about prothrombin activation by the purified venom activator are given in the paragraph on the determination of the kinetic parameters of venom-catalyzed prothrombin activation (see below).

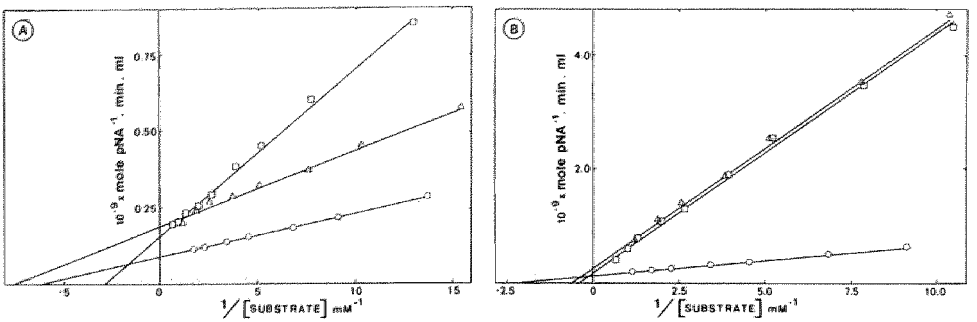

Fig. 2. **Lineweaver-Burk plots of chromogenic substrate conversion by Factor Xa and the venom activator from Oxyuranus scutellatus.** Chromogenic substrate conversion by 0.5 nM Factor Xa (A) or 1.1 nM purified venom activator (B) was measured at 37 °C in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, and 0.5 mg/ml HSA. The chromogenic substrates used were S2222 (□-□), S2337 (△-△), and the Pentapharm Factor Xa substrate CH_3OCO-D-CHG-Gly-Arg-pNA.AcOH (○-○). p-nitroaniline (pNA) formation was measured as described in the experimental procedures. The molar concentration of venom activator in the reaction mixture was calculated from the amount of venom activator protein present, and using a molecular weight of 300,000 for the activator.

Table II

Kinetic parameters for chromogenic substrate conversion by Factor Xa and the venom activator from Oxyuranus scutellatus

Kinetic parameters for chromogenic substrate conversion were obtained from Lineweaver-Burk plots (Fig. 2) after statistical analysis according to Eisenthal and Cornish-Bowden (21).

	Factor Xa			Venom activator		
Chromogenic substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
S2222	346	211	0.6×10^6	2349	83	0.35×10^5
S2337	130	173	1.3×10^6	1531	55	0.36×10^5
Pentapharm[a]	157	376	2.4×10^6	434	124	2.90×10^5

[a]Pentapharm Factor Xa Substrate: CH_3OCO-D-CHG-Gly-Arg-pNa.AcOH

Molecular weight determination of the purified venom activator. Polyacrylamide gradient gel electrophoresis under non-denaturing conditions was performed to establish the molecular weight of the purified activator. After electrophoresis, the gel was sliced, and the gel slices were eluted and assayed for amidolytic activity toward S2337 and prothrombin-converting activity (Fig. 3). It appeared that there were two bands with S2337

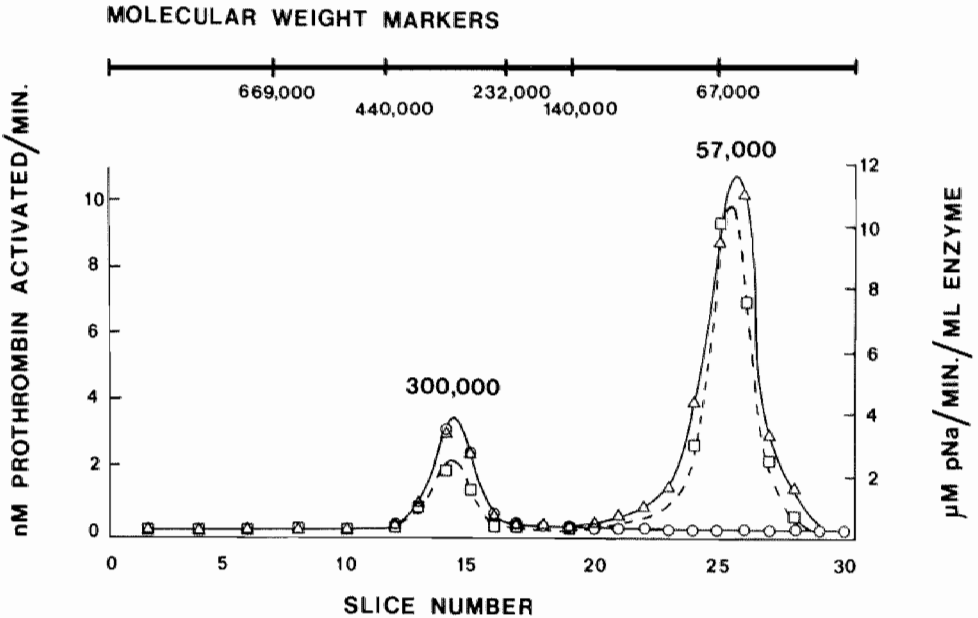

Fig. 3. **Gradient gel electrophoresis of the purified prothrombin activator from Oxyuranus scutellatus.** The purified venom activator (62 µg) was subjected to electrophoresis under non-denaturing conditions on a Pharmacia PAA 4/30 gradient gel. Part of the gel was reserved for the molecular weight markers thyroglobulin (M_r 669,000), ferritin (M_r 440,000), catalase (M_r 232,000), lactate dehydrogenase (M_r 140,000), and bovine serum albumin (M_r 67,000). After electrophoresis, the gel was sliced (2.5 mm slices) and the protein was eluted from the gel by shaking the gel slices for 48 h at 4 °C in 1 ml of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$ containing 0.5 mg/ml HSA. The prothrombin-converting activity in 1 µl of the gel eluate was determined in the absence (○-○) and presence of Factor Va (△-△). The amidolytic activity in 100 µl of eluate was determined with S2337 (□-□). Further details about these assays are given in the experimental procedures. The part of the gel that contained the molecular weight markers was stained with 0.05% Amido Black, and the migration distance of the marker proteins is indicated in the figure.

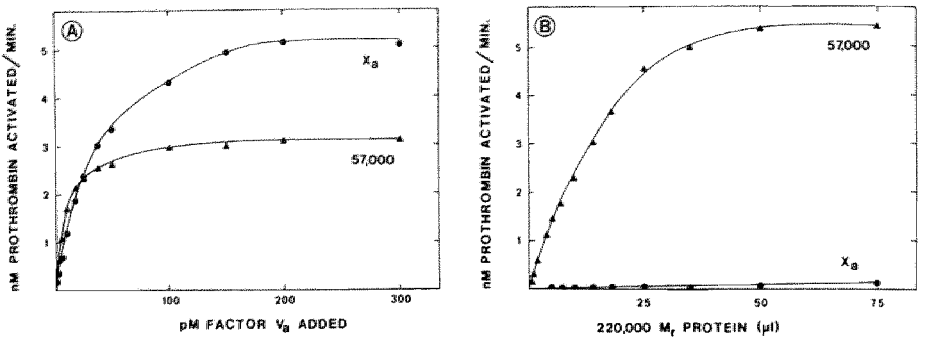

Fig. 4 **Prothrombin activation by the M_r 57,000 subunit of Oxyuranus scutellatus: effect of Factor Va and the M_r 220,000 component.** The eluate of fraction 26 of the gradient gel (Fig. 2) containing the M_r 57,000 subunit of the venom activator was diluted 10-fold, and 20 µl of this dilution were incubated at 37 °C in 180 µl of 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, and 0.5 mg/ml human serum albumin containing different amounts of Factor Va (▲-▲, A) or the eluate of the gel fraction 17 which contains the M_r 220,000 component (▲-▲, B). After 6 min, 20 µl of this mixture were added to 430 µl of the same buffer containing 50 µM phospholipid (PS/PC, 25/75; mole/mole). 5 min later, 50 µl of 5 µM prothrombin was added, and the rate of prothrombin activation was measured as described in the experimental procedures. In a similar experiment, 40 pM Factor Xa was titrated with varying amounts of Factor Va (●-●, A) or M_r 220,000 eluate (●-●, B). The amounts of Factor Va or the M_r 220,000 component indicated in the figure are those present in the final prothrombin activation mixture (Factor Va) or in the preincubation mixture (M_r 220,000 component).

hydrolase activity: one at a molecular weight of about 300,000 and one at 57,000. The prothrombin-converting activity of these two bands was, however, markedly different. The M_r 300,000 protein rapidly activated prothrombin, and its activation rate was not affected by Factor Va. Prothrombin conversion by the M_r 57,000 protein was very slow, but was greatly increased in the presence of bovine Factor Va. In Fig. 4A, it is shown that low amounts of Factor Va were required to obtain maximal prothrombin-converting activity of the M_r 57,000 protein. This indicates that the latter protein has a high affinity for Factor Va. For comparison, a Factor Va titration of a limited amount of Factor Xa is also included in this figure.

Recombinations of gel slice eluates showed that there was another protein band, with a molecular weight of about 220,000, which, like Factor Va, stimulated prothrombin activation by the M_r 57,000 eluate. The M_r 220,000

eluate had no catalytic activity but restored prothrombin activation by the M_r 57,000 protein band in a concentration-dependent manner (Fig. 4B). The M_r 220,000 protein band was, however, not able to stimulate Factor Xa-catalyzed prothrombin activation. Since the experiments presented in Figs. 4 (A and B) were carried out at the same concentration of the M_r 57,000 subunit, it should be noted that saturation with the M_r 220,000 component gives an enzyme-cofactor complex that is twice as active as the complex obtained upon saturation with bovine Factor Va.

When the purified venom activator was subjected to prolonged electrophoresis on the gradient gels, the active species with M_r 300,000 completely disappeared, whereas the activity of the M_r 57,000 component was increased (data not shown). This indicates that the native venom activator is a high molecular weight protein (M_r 300,000) that can dissociate into an enzymatic component (M_r 57,000) with high amidolytic and low prothrombinase activity and a cofactor part (M_r 220,000) which stimulates the prothrombin-converting activity of the enzymatic component.

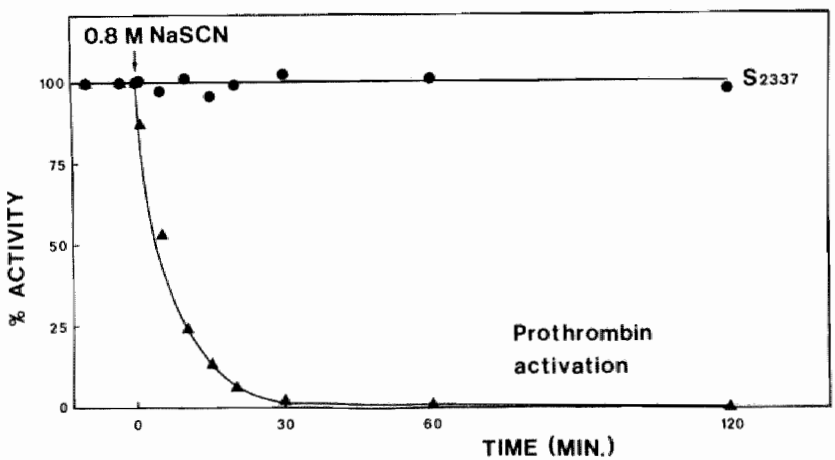

Fig. 5. **Effect of NaSCN on the catalytic activity of the prothrombin activator from Oxyuranus scutellatus.** The purified venom activator was incubated at 37 °C at a concentration of 62 µg/ml in 50 mM Hepes (pH 7.2), 150 mM NaCl, and 0.5 mg/ml human serum albumin. At zero time, NaSCN was added to a final concentration of 0.8 M. At the time intervals indicated, samples from this mixture were diluted and assayed for amidolytic activity toward S2337 (●-●) or prothrombin-converting activity (▲-▲). Further details about the amidolytic assay and the prothrombinase assay, which was carried out in the presence of 50 µM phospholipid, but without Factor Va, are given in the experimental procedures. The low amount of NaSCN present in the diluted venom aliquots did not interfere with these assays.

Treatment of the venom activator with sodium thiocyanate (NaSCN). Since it is likely that the 57,000 protein is derived from dissociation of the native activator, we searched for a way to treat the venom activator so that it would retain its amidolytic activity toward S2337 but would lose its prothrombin-converting activity. Walker et al. (11) have shown that incubation of the venom from the subspecies Oxyuranus scutellatus scutellatus in 2 M NaCl results in a loss of procoagulant activity while the S2222 hydrolase activity remains constant. We observed that the loss of prothrombin-converting activity of the purified venom activator from Oxyuranus scutellatus in the presence of 2 M NaCl is, however, rather slow ($t_{\frac{1}{2}} > 2$ h). The prothrombin-converting activity of the venom activator is rapidly lost upon incubation with 0.8 M NaSCN (Fig. 5). Within 30 min, more than 95% of the venom activity in prothrombin activation was lost. The amidolytic activity toward S2337 was not affected by incubation of the venom activator with NaSCN.

Table III

Effects of phospholipid plus Ca^{2+} and Factor Va on prothrombin activation by the complete purified activator and the NaSCN-treated activator from Oxyuranus scutellatus venom

0.5 μM prothrombin was activated by appropriate dilutions of the purified venom activator or the NaSCN-treated activator in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 0.2 mM EDTA, 0.5 mg/ml HSA either in the absence or in the presence of 5 mM $CaCl_2$, 5 nM Factor Va (Va), and/or 50 μM phospholipid (PL) vesicles containing 25 mol % PS and 75 mol % PC. The NaSCN-treated activator was obtained by incubating the purified venom actvator (62 μg/ml) for 2 h in the presence of 0.8 M NaSCN followed by dialysis against 50 mM Tris-HCl (pH 7.9), 175 mM NaCl. Prothrombin activation was determined as described in the experimental procedures, and the rate measured with the native activator without accessory components, 1.61 nmol prothrombin activated/min/mg venom, was arbitrarely taken as 1.

Activator	Native activator (relative rate)	NaSCN-treated activator (relative rate)
Venom	1	< 0.03
Venom, Ca^{2+}	8	< 0.03
Venom, Ca^{2+}, Va	7	0.35
Venom, Ca^{2+}, PL	3365	1.75
Venom, Ca^{2+}, PL, Va	3487	1789

The prothrombin-converting activity of the NaSCN-treated activator could be restored with bovine Factor Va. This effect is most clearly seen upon comparison of the relative rates of prothrombin activation by the purified activator and the NaSCN-treated activator in the presence of different accessory components (Table III). With both forms of the activator, prothrombin activation was stimulated by phospholipids plus Ca^{2+}. Factor Va, which did not stimulate the native venom activator, drastically increased the rate of prothrombin activation by the NaSCN-treated activator. This rate enhancement by Factor Va was observed in the presence as well as in the absence of phospholipids.

Kinetic parameters of venom-catalyzed prothrombin activation. To gain more insight in the mechanism by which phospholipids plus Ca^{2+} stimulate the catalytic activity of the native venom activator and how Factor Va stimulates the NaSCN-treated activator, we have determined the kinetic parameters of venom-catalyzed prothrombin activation in activation mixtures of different compositions. Prothrombin activation was determined by measuring the generation of amidolytic activity toward S2238. Prothrombin activation was linear with time and proportional with the amount of venom activator present, and Michaelis Menten kinetics was observed. The kinetic parameters were obtained from Lineweaver-Burk plots, a few typical examples of which are shown in Fig. 6. The kinetic parameters for venom-catalyzed prothrombin activation are summarized in Table IV.

Prothrombin activation by the native activator alone was characterized by a very high Km and a relatively high Vmax. The catalytic activity of the native venom activator was hardly affected by Factor Va either in the absence or in the presence of phospholipids. Phospholipids drastically decreased the Km for prothrombin and had only a small effect on the Vmax. To obtain a phospholipid-dependent decrease of the Km, the procoagulant membranes had to contain negatively charged phospholipids such as phosphatidylserine. Prothrombin activation by the native activator was, however, rather insensitive to varying the amount of negatively charged phospholipid in the procoagulant membranes since the same kinetic parameters were found for vesicles containing 5 and 25 mol % phosphatidylserine.

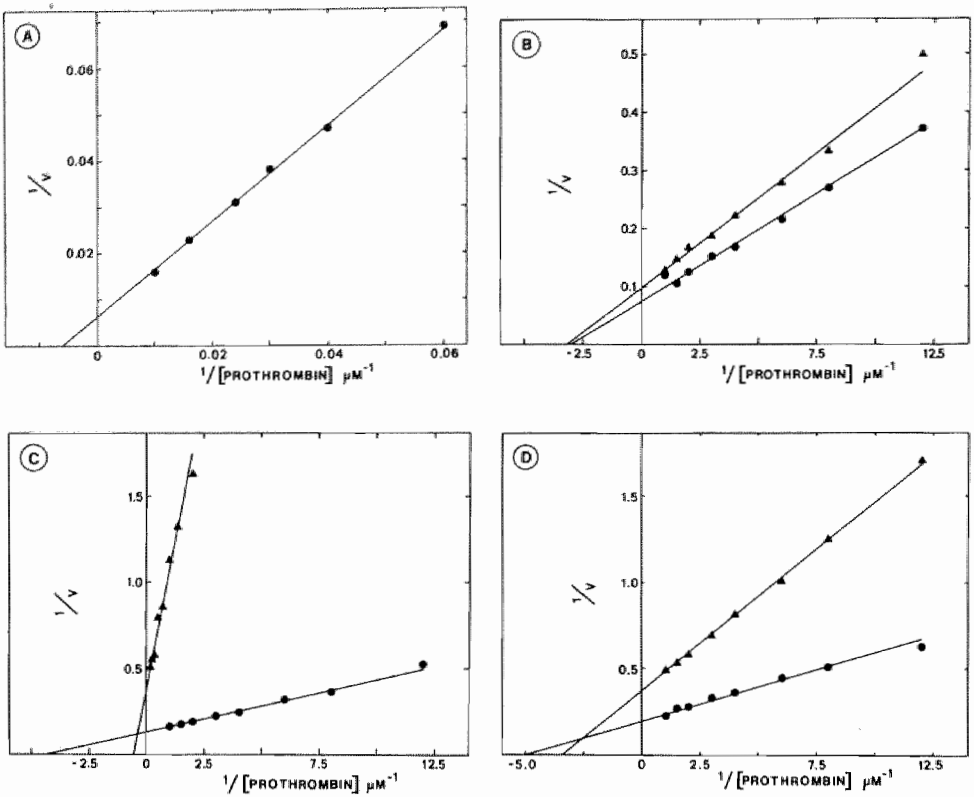

Fig. 6. **Lineweaver-Burk plots of prothrombin activation by the native and NaSCN-treated prothrombin activator from the venom of Oxyuranus scutellatus.** Appropriate dilutions of the purified venom activator or the NaSCN-treated activator (obtained as described in the legend of Table III) were preincubated in 300 µl of a buffer containing 50 mM Tris (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/ml HSA, either with or without phospholipid vesicles and/or factor Va. After 5 min, prothrombin activation was started by the addition of 200 µl of the same buffer (prewarmed at 37 °C) containing various amounts of prothrombin. The rate of prothrombin activation (V, pmoles of prothrombin activated per min per ml reaction mixture) was determined as described in the experimental procedures. The prothrombin-activating complex consisted of:

A) 64 ng/ml native venom activator
B) 1.9 ng/ml native venom activator and 50 µM phospholipid vesicles, containing 25% PS/75% PC (●-●) or 5% PS/95% PC (▲-▲)
C) 1.7 µg/ml NaSCN-treated activator and 50 µM phospholipid vesicles, containing 25% PS/75% PC (●-●) or 5% PS/95% PC (▲-▲)
D) 1.7 ng/ml NaSCN-treated activator, 5 nM factor Va, and 50 µM phospholipid vesicles containing 25% PS/75% PC (●-●) or 5% PS/95% PC (▲-▲)

Table IV

Kinetic parameters of prothrombin activation by the native and NaSCN-treated prothrombin activator from Oxyuranus scutellatus venom

The kinetic parameters Km and Vmax were obtained from Lineweaver-Burk plots after statistical analysis according to Eisenthal and Cornish-Bowden (21). Typical examples of Lineweaver-Burk plots are shown in Fig. 6. The final prothrombin activation mixture contained 50 mM Tris (pH 7.9), 175 mM NaCl, 0.5 mg/ml HSA, varying amounts of prothrombin, appropriate dilutions of the venom activator, 5 mM $CaCl_2$, and, when indicated below, 5 nM Factor Va (Va) and/or 50 µM phospholipid vesicles (25% PS/75% PC or 5% PS/95% PC).

	Native activator		NaSCN-treated activator	
Activating complex	Km(µM)	Vmax[a]	Km(µM)	Vmax[a]
Venom, Ca^{2+}	166	750	n.d.[b]	n.d.
Venom, Ca^{2+}, Va	119	550	n.d.	n.d.
Venom, Ca^{2+}, PS/PC(5/95)	0.32	1582	2.00	0.5
Venom, Ca^{2+}, PS/PC(5/95), Va	0.25	1442	0.29	458
Venom, Ca^{2+}, PS/PC(25/75)	0.34	2137	0.23	1.3
Venom, Ca^{2+}, PS/PC(25/75),Va	0.31	2198	0.20	869

a) Vmax is expressed in moles of prothrombin activated per minute per mole of venom. The molar venom concentration was calculated from the amount of purified venom activator protein using a molecular weight of 300,000.
b) n.d., not determined

We were not able to determine the kinetic parameters of prothrombin activation by the NaSCN-treated activator in the absence of phospholipids since the reaction rates were too low to be measured. However, the kinetic parameters determined in the presence of phospholipids yield sufficient information to gain insight into the changes in the catalytic properties for the NaSCN-treated activator. The NaSCN-treated activator had a low Vmax for prothrombin activation and had an increased Km for prothrombin when procoagulant membranes with a low percentage of phosphatidylserine were part of the prothrombin-activating complex. The kinetic parameters of the NaSCN-treated activator are affected by Factor Va which causes a considerable increase in the Vmax and a drastic decrease in the Km for

prothrombin when phospholipid vesicles with a low mole percent phosphatidylserine were used. Such effects of Factor Va on the Vmax (1,2) and Km (4,5) have also been observed for Factor Xa-catalyzed prothrombin activation. The kinetic properties of the non-treated and NaSCN-treated venom activator suggest that the native activator is comprised of a Factor Xa-like catalytic unit associated with a large molecular weight cofactor with Factor Va-like function. Upon treatment with NaSCN, the cofactor function is lost, which results in a decreased catalytic capacity that can subsequently be restored with Factor Va. Factor Va is, however, not able to fully restore the prothrombin-converting activity of the activator. This indicates that the venom's own cofactor is more active than Factor Va (see also Fig. 4, A and B).

Peptide bond cleavage patterns during venom-catalyzed prothrombin activation. Since several peptide bonds in prothrombin are susceptible to proteolytic cleavage, different activation products can accumulate during prothrombin activation (Fig. 7). Cleavage of peptide bonds 1 and 2 can be catalyzed by Factor Xa and can result in the production of prethrombin 2 plus fragment 1.2 (bond 1), meizothrombin (bond 2), or thrombin plus fragment 1.2 (bonds 1 and 2). Both meizothrombin and thrombin have their active site serine exposed and can cleave the chromogenic substrate S2238, whereas prethrombin 2 has no enzymatic activity. Thrombin can cleave peptide bond 3 and produce prethrombin 1 plus fragment 1.

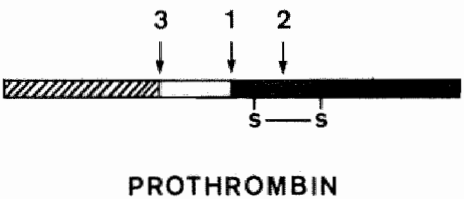

Fig. 7. Peptide bonds in prothrombin susceptible to proteolytic cleavage.

Fig. 8 shows a gel electrophoretic analysis of the time course of prothrombin cleavage by the native venom activator in the presence of Ca^{2+} plus phospholipid. This experiment was carried out in the presence of the reversible thrombin inhibitor I2581 in order to slow down feedback

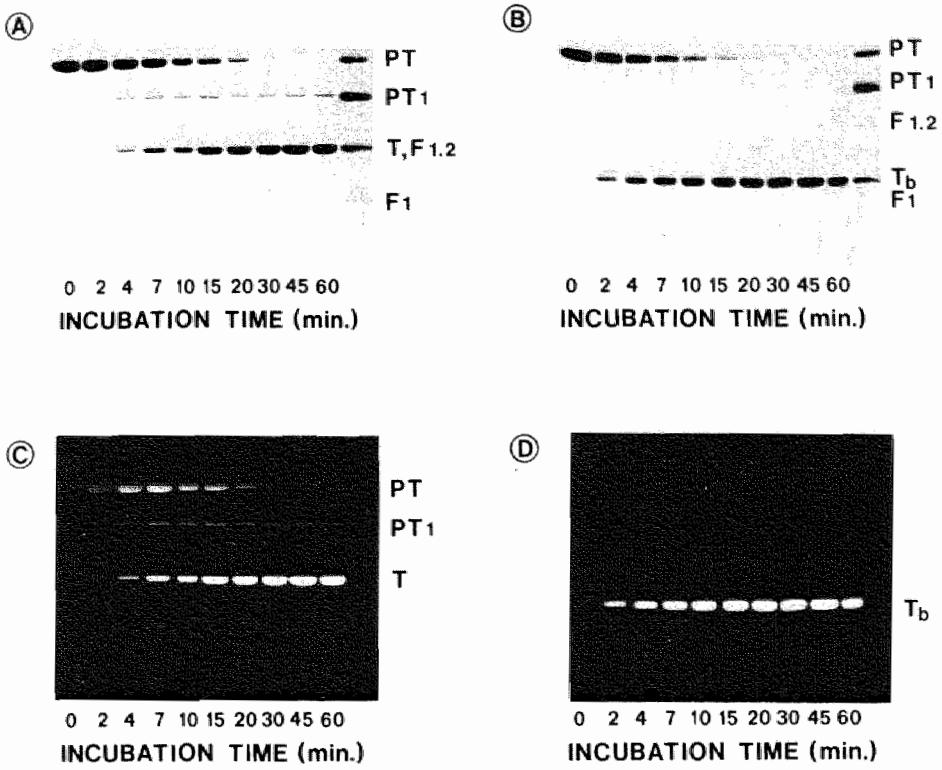

Fig. 8. **Gel electrophoretic analysis of prothrombin activation by the native purified venom activator.** 3 μM prothrombin was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 μM I2581, 5 mM $CaCl_2$, 0.1 μg/ml purified activator, and 50 μM phospholipid (25% PS/75% PC) at 37 °C. At the time intervals indicated, aliquots (100 μl) of this reaction mixture were added to 37.5 μl of gel buffer containing 250 mM Tris-HCl (pH 6.9), 5% SDS, 40 μM iodoacetamide, and 50% (v/v) glycerol or to 12.5 μl of 2 mM dansyl-Glu-Gly-Arg-CH_2Cl in 10 mM HCl. The latter samples were incubated for 15 min at 37 °C, and then 37.5 μl of the gel buffer described above were added. Prior to electrophoresis, the samples were kept for 1 h at 37 °C during which period 5% (v/v) β-mercaptoethanol was present in the disulfide-reduced samples. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (A and B) or transilluminated with long wave ultraviolet light and photographed using a yellow filter and Polaroid type 55 film (C and D). Non-reduced samples are shown in A and C, whereas reduced samples are shown in B and D. The lanes at the far right of the Coomassie Blue-stained gels contain a reference mixture of prothrombin and activation products. PT = prothrombin, PT1 = prethrombin 1, F1.2 = fragment 1.2, PT2 = prethrombin 2, T = thrombin, T_B = B-chain of thrombin and F1 = fragment 1.

reactions of thrombin on prothrombin. The non-reduced gel shows that thrombin is the major reaction product and that small amounts of prethrombin 1 and fragment 1 are formed (Fig. 8A). On the reduced gel, the pattern is somewhat different (Fig. 8B). The intensity of the protein band corresponding to thrombin (the thrombin B-chain) is increased, whereas the intensities of the prothrombin and prethrombin 1 bands are decreased. This indicates that, in addition to thrombin, meizothrombin and meizothrombin des-fragment 1 are also formed. The presence of meizothrombin in the prothrombin activation mixtures becomes even more clear upon electrophoresis of reaction aliquots that were treated with dansyl-Glu-Gly-Arg-CH_2Cl. At high concentrations, this inhibitor alkylates the histidine of the catalytic triad of thrombin, meizothrombin, and meizothrombin des-fragment 1 (22). Due to the fluorescent properties of dansyl-Glu-Gly-Arg-CH_2Cl, the proteins that incorporate this inhibitor can be visualized on the gel after electrophoresis (19). Indeed, Fig. 8C shows that, in aliquots of reaction mixtures in which prothrombin is activated by the venom activator, dansyl-Glu-Gly-Arg-CH_2Cl incorporates into proteins co-migrating with prothrombin, prethrombin 1, and thrombin. Upon reduction all fluorescent label co-migrates with the B-chain of thrombin which indicates that the fluorescent proteins on the non-reduced gels are indeed meizothrombin, meizothrombin des fragment 1 and thrombin (Fig. 8D).

For the NaSCN-treated activator, the cleavage pattern is somewhat different. In addition to meizothrombin, meizothrombin des-fragment 1, and thrombin, there are considerable amounts of prethrombin 2 visible on the gel (Fig. 9). With respect to the comparison of the NaSCN-treated and the native venom activator with Factor Xa and the Factor Xa-Va complex, it is interesting to note that prethrombin 2 is also produced in large amounts when Factor Xa is allowed to activate prothrombin in the absence of Factor Va (1).

Determination of γ-carboxyglutamic acid residues in the venom prothrombin activator. In a previous paper, we have shown that the prothrombin activator from the venom of Notechis scutatus scutatus contains several γ-carboxyglutamic acid (Gla) residues (9). Therefore, we decided to test whether the activator from Oxyuranus scutellatus also contains such residues. The Gla content of the venom activator was determined by high performance liquid chromatography according to Kuwada and Katayama (23). As a control,

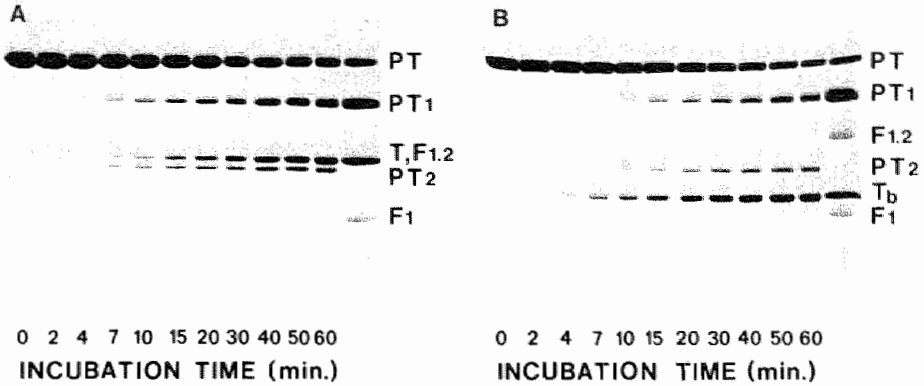

Fig. 9. **Gel electrophoretic analysis of prothrombin activation by the NaSCN-treated venom activator.** The purified venom activator (67.5 µg/ml) was incubated for 1.5 h at 37 °C in the presence of 0.8 M NaSCN, and was subsequently dialyzed against 50 mM Tris-HCl (pH 7.9) and 175 mM NaCl to remove the NaSCN. Prothrombin (6.25 µM) was activated by 56.7 µg/ml of the NaSCN-treated and dialyzed venom activator in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 50 µM phospholipids (25% PS/75% PC), and 20 µM I2581. Gel electrophoretic analysis of prothrombin activation was further carried out as described in the legend to Fig. 8.

we also measured the content of Gla residues in Factor Xa. The chromatograms of alkaline hydrolysates of Factor Xa and of the venom prothrombin activator from Oxyuranus scutellatus are shown in Fig. 10. The peaks corresponding to Gla and Glu are indicated in the figure. The amino acids eluting at 13.88 min (Fig. 10A) and 13.45 min (Fig. 10B) are identified as Gla since they are absent in acid hydrolysates (16 h in 6 N HCl at 100 °C in vacuo) of Gla-containing proteins. Factor Xa had a Gla/(Glu+Gln) ratio of 0.42 which corresponds to about 11 Gla residues/mole since the (Glu+Gln) content of Factor Xa is 25/mole (24). This value is in close agreement with that reported in the literature (12 Gla residues/ Factor Xa, cf.ref. 25). The Gla/(Glu+Gln) ratio observed for the venom activator was 0.042. Although considerably lower than that of Factor Xa this is not surprising since the molecular weight of the venom activator is about 7x higher than that of Factor Xa. Since the (Glu+Gln) content of the venom activator is not known, it is not possible to calculate the exact number of Gla residues. Considering the large molecular weight of the activator, it is, however, likely that the venom activator contains

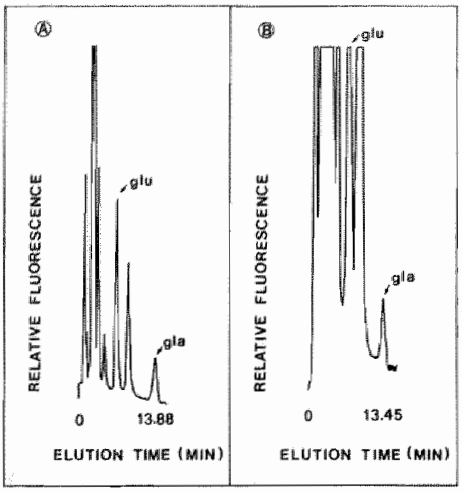

Fig. 10. **HPLC determination of γ-carboxyglutamic acid residues in the venom prothrombin activator from Oxyuranus scutellatus.** The presence of γ-carboxyglutamic acid (Gla) and glutamic acid (Glu) residues in alkaline hydrolysates of Factor Xa (Fig. 10A) or the venom activator (Fig. 10B) were determined on a Nucleosil-5SB column according to the method of Kuwada and Katayama (23). The peaks corresponding with the elution of Glu and Gla are identified in the figure.

sufficient Gla residues to support Ca^{2+}-dependent binding of the activator to negatively charged phospholipid surfaces.

Comparison of functional properties of prothrombin activators in venoms from Australian elapid snakes. It is generally believed that the coagulant action of Australian elapid venoms is mainly due to the presence of prothrombin activators that require cofactors for their activity (27). Only the venom activators from Notechis scutatus scutatus and Oxyuranus scutellatus have so far been studied in sufficient detail to allow conclusions regarding their mode of action in prothrombin activation. To test whether the other Australian elapid venoms contain a Notechis-like prothrombin activator that is stimulated by both phospholipids and Factor Va or whether they contain an Oxyuranus-like prothrombin activator that is stimulated by phospholipids and that has its own Factor Va-like cofactor, we have compared the prothrombin-converting properties of a number of commercially available venoms (Table V). The experiment reported in this table was carried out as follows. Amounts of venom from the different snakes were taken that gave approximately the same rate of prothrombin activation without added accessory components. The stimulatory effect of phospholipid and phospholipid plus Factor Va on the venom activity was measured to determine the cofactor dependence of venom-catalyzed prothrombin activation. Finally, the venoms were treated with NaSCN to test

Table V

Effects of phospholipids and Factor Va on prothrombin activation by various snake venoms

Prothrombin activation was determined in a reaction mixture containing 0.5 μM prothrombin, appropriate dilutions of crude venom, 5 mM $CaCl_2$, with or without 50 μM phospholipid (PL) or 50 μM phospholipid plus 5 nM Factor Va (Va) in 50 mM Tris (pH 7.9), 175 mM NaCl and 0.5 mg/ml HSA. The phospholipid vesicles contained 25% PS and 75% PC. Prothrombin activation was determined as described in the experimental procedures. The rate of prothrombin activation measured in the absence of phospholipids and Factor Va is expressed as nanomoles of prothrombin activated per min per mg of crude venom. The stimulation by PL or PL + Va is obtained by comparison with the rate of prothrombin activation in the absence of accessory components which for each individual venom was arbitrarily taken as 1.

Venom	nmol prothrombin actived/min/mg venom without PL and Va	Stimulation Factor	
		PL	PL + Va
Notechis scutatus scutatus	0.27×10^{-3}	7570	34.2×10^{6}
Notechis ater niger	0.24×10^{-3}	1453	5.4×10^{6}
Notechis ater humphreysi	0.14×10^{-3}	6422	13.5×10^{6}
Tropidechis carinatus	0.08×10^{-3}	3260	9.1×10^{6}
Pseudechis porphyriacus	0.11×10^{-3}	1443	2.7×10^{6}
Hoplocephalus stephensii	0.07×10^{-3}	400	6.3×10^{6}
Oxyuranus microlepidotus	0.48	527	713
Oxyuranus scutellatus	2.13	448	456
Pseudonaja textilis textilis	11.4	257	297
Dispholidus typus	241	0.7	0.7
Echis carinatus	19	0.8	1.4

whether they lost prothrombin-converting activity that could be restored with Factor Va, a property which would indicate that the venom activator, like Oxyuranus, contains a Factor Va-like cofactor. Table V shows that the Australian elapid venoms can indeed be divided in two groups: one group containing a Notechis-like prothrombin activator (Notechis scutatus scu-

tatus, Notechis ater niger, Notechis ater humphreysi, Tropidechis carinatus, Pseudechis porphyriacus, and Hoplocephalus stephensii) which requires phospholipids plus Ca^{2+} and Factor Va and a second group of Oxyuranus-like prothrombin activators (Oxyuranus scutellatus, Oxyuranus microlepidotus and Pseudonaja textilis textilis) which require phospholipids plus Ca^{2+} but are not stimulated by Factor Va. The venoms of this group lose their ability to activate prothrombin upon NaSCN treatment, and rapid prothrombin activation by the NaSCN-treated venom becomes dependent upon the presence of Factor Va (Table VI). This indicates that this group of venoms indeed contains a prothrombin activator comprised of a catalytic unit and a Factor Va-like cofactor. For comparison, the data obtained with a number of venoms not belonging to the Australian Elapidae are also given in Tables V and VI. The well-known prothrombin activators from Echis carinatus and Dispholidus typus are not affected by the accessory components of the prothrombinase complex.

Table VI

Effect of Factor Va on prothrombin activation by crude venoms treated with NaSCN

Crude venoms of various snakes were incubated at 37 °C for 24 hrs at 0.1 mg/ml in 50 mM Tris (pH 7.9), 175 mM NaCl, with or without 0.8 M NaSCN. The prothrombin-converting activity of the non-treated and NaSCN-treated venoms were determined in the presence of phospholipid with and without Factor Va as described in the experimental procedures. c* represents the ratio of the stimulatory effect of Factor Va on the rate of prothrombin activation by the NaSCN-treated and non-treated venom.

Venom	c	Venom	c
Notechis scutatus scutatus	1.3	Oxyuranus scutellatus	1933
Notechis ater niger	1.3	Oxyuranus microlepidotus	981
Notechis ater humphreysi	1.1	Pseudonaja textilis textilis	262
Tropidechis carinatus	1.2		
Pseudechis porphyriacus	1.1	Dispholidus typus	2.6
Hoplocephalus stephensii	n.d.**	Echis carinatus	1.9

* $c^{*} = \dfrac{(V_{\text{prothrombin activation+Va}}/V_{\text{prothrombin activation-Va}})\ \text{NaSCN-treated venom}}{(V_{\text{prothrombin activation+Va}}/V_{\text{prothrombin activation-Va}})\ \text{non-treated venom}}$

** The prothrombin-converting activity was completely lost upon NaSCN treatment and was not restorable with Factor Va

DISCUSSION

Prothrombin activators from the venoms of the Australian Elapidae share many properties with Factor Xa, the physiological activator of prothrombin. One of the major similarities with Factor Xa concerns the observation that the prothrombin-converting activity of the venom activators can also be stimulated by the accessory components (phospholipids and/or Factor Va) of the prothrombinase complex. In this paper, we have described the purification and the structural and functional characterization of the prothrombin activator from the venom of Oxyuranus scutellatus (Taipan snake). The prothrombin-converting activity of this venom activator is drastically enhanced by the presence of phospholipids plus Ca^{2+} but is not affected by Factor Va. The native activator is a large protein molecule with an apparent molecular weight of about 300,000 which appears to consist of a catalytic unit of M_r 57,000 bearing the active site and a cofactor part with a Factor Va-like function, which has an apparent M_r of 220,000. Walker et al. (11) suggested that the venom prothrombin activator from Oxyuranus scutellatus scutellatus may be comprised of either two enzymes or a single enzyme bearing two catalytic sites with distinct activities. Our data show that the venom activator is actually one enzyme that can exist in two forms, i.e. one form with and one form without protein cofactor.

Several observations reported in this paper indicate that the mode of action of this cofactor part in venom-catalyzed prothrombin activation is similar to that of Factor Va in prothrombin activation by Factor Xa. The catalytic properties of the native venom activator resemble those of the Factor Xa-Va complex. Both catalyze prothrombin activation with a high Vmax and for both enzyme complexes, the Km for prothrombin is high. The Km decreases considerably when negatively charged phospholipids are introduced as part of the prothrombin-activating complex. The native activator also shares the property of the Factor Xa-Va complex in that low Km values for prothrombin are also measured when phospholipids with a low affinity for prothrombin (i.e. membranes with a low mole percentage of PS) are used as procoagulant surface. Detailed kinetic studies of prothrombin activation by the Factor Xa-Va complex have shown that this phenomenon is caused by Factor Va (5).

The main support for the hypothesis that the venom prothrombin activator from Oxyuranus scutellatus is a multimeric protein consisting of a catalytic unit associated with a Factor Va-like cofactor came from an experiment in which the native activator was subjected to prolonged gradient gel electrophoresis under non-denaturing conditions. In this experiment, the catalytic and cofactor parts of the venom activator were separated from each other and appeared in different parts of the gel. The catalytic unit migrated at a molecular weight of 57,000 and had a low prothrombin-converting activity and a high amidolytic activity. The prothrombin-converting activity of this low M_r catalytic unit could be restored with bovine Factor Va and with a protein that could be eluted from the gradient gel, which had an apparent molecular weight of 220,000. This protein is presumably the Factor Va-like cofactor part of the venom activator.

The native venom activator also structurally resembles the Factor Xa-Va complex. The catalytic unit of the venom activator consists of two polypeptide chains of M_r 30,000 linked together via a disulfide bridge. Factor Xa is also a two-chain molecule. It consists of a heavy chain of M_r 30,000 which bears the catalytic site and which is linked via a disulfide bridge to a light chain of M_r 15,000. Gel electrophoretic analysis of the purified venom activator shows two other major protein bands with apparent molecular weights of 110,000 and 80,000. They likely comprise the cofactor part of the venom activator and may structurally resemble Factor Va. Bovine Factor Va has a molecular weight of 180,000 and consists of two subunits of M_r 115,000 and 73,000 (26). The structural features of the venom prothrombin activator led us to the hypothetical model shown in Fig. 11. We propose that the Oxyuranus activator consists of a Factor Xa-like part consisting of two subunits of M_r 30,000 and a Factor Va-like part of about M_r 220,000 that is composed of two polypeptide chains of approximately M_r 110,000 and 80,000. Since the apparent molecular weights of the native activator (300,000) and the cofactor part (220,000) are somewhat higher than the sum of the individual subunits, it is possible that some low M_r subunits that may have escaped our attention are also part of the native activator. The hypothetical model does not explain the chemical or physical nature of the forces that are responsible for the association of the subunits within the cofactor part. It is possible that in a fraction of the activator

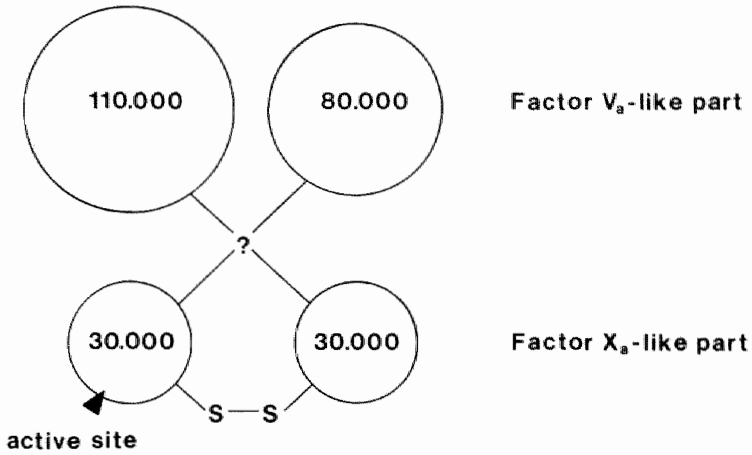

Fig. 11. **Hypothetical model for the venom prothrombin activator from Oxyuranus scutellatus.**

molecules, these subunits are held together by a disulfide bridge since on non-reduced SDS gels a subunit of M_r 220,000 is observed. Upon reduction, this band disappears and dissociates into subunits that co-migrate with the M_r 110,000 and 80,000 polypeptides. The polypeptide subunits that we observed to be present in the prothrombin activator preparation from Oxyranus scutellatus was somewhat different from the subunit composition of the activator isolated from Oxyuranus scutellatus scutellatus by Walker et al. (11). They reported that their activator preparation contains two bands with molecular weights corresponding to 220,000 and 160,000 which upon reduction separate into two bands of M_r 105,000 and 76,000. We did not observe a M_r 160,000 subunit on non-reduced gels, whereas Walker et al. (11) did not identify the presence of the weakly staining M_r 60,000 subunit (M_r 30,000 on reduced gels) that contains the catalytic site of the activator. The discrepancy with regard to the M_r 160,000 subunit on the non-reduced SDS gel may be due to the use of venom preparations from different subspecies of Oxyuranus scutellatus or to the earlier mentioned possibility of partial reduction of disulfide bridges that may have occurred in our preparation.

Finally, it is important to mention that the venom activator, like Factor Xa, contains γ-carboxyglutamic acid residues. Although their exact number is not known, it is likely that sufficient residues are present to support

the Ca^{2+}-dependent binding of the venom activator to negatively charged phospholipids. Such binding would explain their stimulatory effect on venom-catalyzed prothrombin activation as well as on the corresponding phospholipid-dependent decrease in the Km for prothrombin. It is likely that these effects are caused by simultaneous binding of the venom activator and the substrate prothrombin to the phospholipid surface. Such simultaneous binding will promote the formation of the enzyme-substrate complex and enhance the rate of prothrombin activation (cf. refs. 1 and 5 for a detailed description of such a model for phospholipid involvement in prothrombin activation by Factor Xa).

The presence of a complex prothrombin activator in a snake venom is not typical for Oxyuranus scutellatus. In Table VI it is shown that the catalytic properties of a number of other Australian Elapidae (Pseudonaja textilis textilis and Oxyuranus microlepidotus) show a high degree of similarity with the activator from Oxyuranus scutellatus. These venoms presumably also contain a multimeric prothrombin activator comprised of an enzyme associated with a Factor Va-like cofactor that greatly enhances the catalytic capacity of these venom activators.

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CHAPTER IV

PLATELET PROCOAGULANT PROPERTIES STUDIED WITH SNAKE VENOM PROTHROMBIN ACTIVATORS

SUMMARY

Purified snake venom prothrombin activators were used to probe the procoagulant properties of platelet membranes. Human platelets were able to stimulate prothrombin activation by the venom activators from Oxyuranus scutellatus and Notechis scutatus, whereas the prothrombin activator from Echis carinatus was not affected by the presence of platelets. The prothrombin-converting activity of platelets was further studied with the venom activator from Oxyuranus scutellatus and with the Factor Xa-Va complex as prothrombin-activating enzymes. Stimulation of platelets with collagen, collagen plus thrombin, or with the Ca-ionophore A23187 resulted in a considerable increase of platelet prothrombin-converting activity probed with the Factor Xa-Va complex, as well as with the prothrombin activator from Oxyuranus scutellatus. The stimulatory effect of activated platelets on the rates of prothrombin activation by Oxyuranus scutellatus was similar to that determined for Factor Xa-Va-catalyzed prothrombin activation. Compared to non-stimulated platelets, platelets stimulated with thrombin plus collagen exposed twenty times more procoagulant sites for the Factor Xa-Va complex, as well as for the venom activator from Oxyuranus scutellatus. The actual number of procoagulant sites per platelet determined with the Factor Xa-Va complex was in close agreement with the number of sites determined with the venom activator. The time course of appearance of procoagulant activity during platelet stimulation by collagen plus thrombin was also comparable for both activator complexes. Phospholipase A_2 treatment of stimulated platelets resulted in an almost complete loss of their ability to stimulate prothrombin activation by the enzyme from Oxyuranus scutellatus or by the Factor Xa-Va complex. The findings presented in this paper suggest that a) the Factor Xa-Va complex and the prothrombin activator from Oxyuranus scutellatus recognize the same

procoagulant sites on both stimulated and unstimulated platelets, and b) negatively charged phospholipids are essential components of these procoagulant sites.

INTRODUCTION

Blood platelets play an important role in the interactions and reactions between coagulation factors that are essential for hemostatic plug formation. It has been shown that activated platelets stimulate prothrombin and Factor X activation (1,2). Several studies indicate that platelets have the ability to bind the participating coagulation factors, thus promoting the assembly of the prothrombin- and Factor X-activating complexes and enhancing the rate of coagulation factor activation (3-6). The chemical nature of the platelet procoagulant sites is still a matter of debate. It has been proposed that specific protein receptors may participate in the binding of coagulation factors to the platelet plasma membrane (7-9). However, at present there is no direct evidence for the existence of such proteins. Another suggestion is that negatively charged phospholipids, especially phosphatidylserine, are involved in the binding of coagulation factors to the platelet membrane (10,11). This hypothesis is based on two observations: 1) in vitro prothrombin and Factor X activation is stimulated by membrane surfaces that contain negatively charged phospholipids (12), and 2) activation of platelets leads to exposure of phosphatidylserine residues in the outer leaflet of the platelet plasma membrane with a concomitant increase of platelet prothrombin- and Factor X-converting activity (6,10). It should be emphasized, however, that these two models do not exclude each other, and that it is possible that both protein and lipid components may function in the procoagulant sites that are involved in prothrombin and Factor X activation.

In an attempt to gain more insight in the nature of these binding sites, we have used purified snake venom prothrombin activators. Venom activators from different snake species have widely different properties in prothrombin activation (13). It has been shown that there is a group of venom activators that has no cofactor requirement (14,15), whereas there are snake venoms with a prothrombin activator, which is stimulated either

by phospholipids alone (16,17) or by phospholipids and Factor Va (18,19). In the present study, we show that prothrombin activation by the venom activators from Notechis scutatus (Tiger Snake) and Oxyuranus scutellatus (Taipan Snake) is strongly stimulated by blood platelets. Since the venom activator from Oxyuranus scutellatus is only stimulated by negatively charged phospholipids, and not by Factor Va, it was studied in more detail, and its platelet-dependent prothrombin-converting activity was compared with that of the Factor Xa-Va complex. Our results indicate that the functional binding sites for the Oxyuranus scutellatus activator on platelets are the same sites, which participate in prothrombin activation by the Factor Xa-Va complex, and that negatively charged phospholipids are an essential component of these sites.

MATERIALS AND METHODS

Reagents. S2238 was purchased from AB Kabi Diagnostica. Dansyl-Glu-Gly-Arg-CH_2Cl and the calcium ionophore A23187 were obtained from Calbiochem. p-NPGB was from ICN Nutritional Biochemicals. DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-100 and Sephadex G-200 were products of Pharmacia. Ovalbumin, human serum albumin (fatty acid free), benzamidine-HCl, 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) and lyophilized crude snake venoms of Notechis scutatus and Oxyuranus scutellatus were obtained from Sigma. Ecarin, the purified prothrombin activator from Echis carinatus venom, and CH_3OCO-D-CHG-Gly-Arg-pNA.AcOH, a chromogenic substrate for Factor Xa, were purchased from Pentapharm Ltd. Naja naja venom was from Koch Light. Horse tendon collagen was purchased from Hormon Chemie. Teflon-coated magnetic stirring bars, 7x2 mm, were from Bel-Art Products Pequannock, N.J.. All reagents were of the highest grade commercially available.

Proteins. The prothrombin activators from Notechis scutatus and Oxyuranus scutellatus were purified from their respective crude venoms as described earlier (17,19). Phospholipase A_2 was purified from Naja naja venom by the method of Zwaal et al. (20). Bovine prothrombin was purified as described by Owen et al. (21). Bovine Factor Xa was purified according to the method

of Fujikawa et al. (22). Thrombin was purified as described before (23). Factor V and Factor Va were obtained as described by Lindhout et al (24). The protein preparations were stored at -80 °C in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 0.5 mg/ml human serum albumin. Factor Va was stored in the same buffer containing 5 mM $CaCl_2$. Phospholipase A_2 was stored in 50% glycerol at -20 °C.

Protein concentrations. The concentrations of thrombin (25) and Factor Xa (26) were determined by active site titration with p-NPGB. Prothrombin concentrations were obtained with the same method after complete activation with Echis carinatus venom. Factor Va concentrations were determined by kinetic analysis as described by Lindhout et al. (24). The concentration of the purified activator from Notechis scutatus was calculated from a protein determination according to Lowry et al. (27), using bovine serum albumin as a standard, and assuming a molecular weight of 54,000 for the venom activator (19). The concentration of the purified Oxyuranus scutellatus activator was established by titration with dansyl-Glu-Gly-Arg-CH_2Cl (cf. ref. 28), an irreversible inhibitor of Factor Xa-like enzymes. The concentration of Oxyuranus scutellatus obtained in this way correlated well with the concentration calculated from a protein determination according to Sedmak et al. (29), and using a molecular weight of 300,000 for the venom activator (17).

Phospholipids and phospholipid vesicle preparations. 1,2-Dioleoyl-sn-glycero-3-phosphoserine (PS) was prepared from PC by enzymatic synthesis (30). Single bilayer phospholipid vesicles were prepared by sonication of a mixture of 10 % PS and 90 % PC (mol/mol) as described earlier (23). Phospholipid concentrations were determined by phosphate analysis (31).

Isolation of platelets. Human platelets from healthy volunteers were isolated by differential centrifugation as described before (6). No loss of platelet procoagulant activity was found for at least 15 hours when platelets were kept at room temperature in a buffer containing 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, and 0.5 mg/ml human serum albumin (pH 7.5). Platelet concentrations were measured with a Coulter counter (Coulter Electronics).

Platelet stimulation and determination of prothrombin-converting activity. The effect of platelets in prothrombin activation was determined in plastic flat-bottom tubes (2 ml), in which the reaction mixtures were stirred at 350 rpm with teflon stirring bars. To a reaction tube, containing 292.5 µl of a platelet suspension, 13 µl of 75 mM $CaCl_2$ were added. The tube content was warmed and stirred at 37 °C. Platelet activation was started after 5 minutes by addition of a platelet stimulator, resulting in a final volume of 325 µl and a $CaCl_2$ concentration of 3 mM. After a variable time period, referred to as platelet activation time, the platelet prothrombin-converting activity was determined as described below.

Assay of platelet activity in prothrombin activation. 30 to 120 seconds before measuring the effect of platelets in prothrombin activation, 50 µl of prothrombin activator (Factor Xa with or without Factor Va, or venom activator with or without Factor Va) were added to the platelet suspension. Prothrombin activation was started by adding 125 µl of prewarmed prothrombin in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 15.2 mM $CaCl_2$ and 0.5 mg/ml human serum albumin. The final reaction mixture (500 µl) contained 20 mM Tris, 6 mM Hepes, 150 mM NaCl, 1.6 mM KCl, 1.2 mM $MgCl_2$, 6 mM $CaCl_2$, 2.9 mM glucose, 0.5 mg/ml human serum albumin at pH 7.9, and amounts of platelets, prothrombin, prothrombin activator, and Factor Va as indicated in the legends to the tables and figures. Aliquots (usually 5 to 10 µl) were taken from the reaction mixture 10 and 20 seconds after the onset of prothrombin activation. These aliquots were transferred to cuvettes, containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin, and 235 µM S2238 in a final volume of 1 ml. The conversion of S2238 was followed by measuring the absorbance change on an Aminco DW-2C spectrophotometer set in the dual wavelength mode at 405-500 nm. The amount of prothrombin that is activated in the reaction mixture was calculated from a calibration curve, made with known amounts of active site-titrated thrombin.

RESULTS

Cofactor dependence of venom prothrombin activators. Snake venom prothrombin activators may have different cofactor requirements to express

maximal prothrombin-converting activity. To obtain information about the effect of cofactors on various venom activators, prothrombin activation rates were determined as a function of the concentration of phospholipid (10% PS, 90% PC; mol/mol) in the absence as well as in the presence of bovine Factor Va (Fig. 1). In order to saturate all procoagulant sites that are available on the lipid surface, the experiments have been carried out at high concentrations of coagulation factors and venom enzymes.

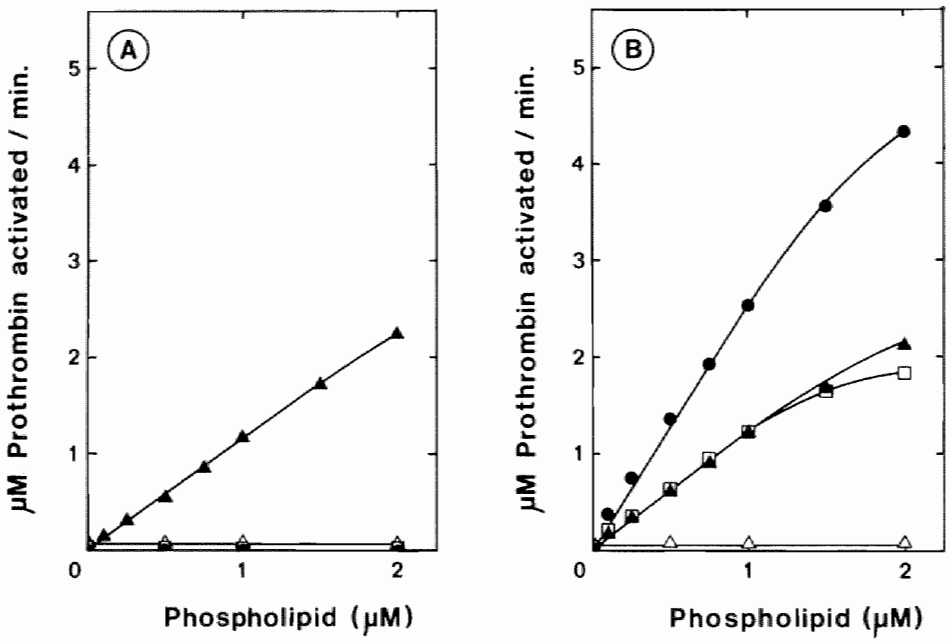

Fig. 1. **Effect of phospholipid and Factor Va on rates of prothrombin activation by Factor Xa and venom prothrombin activators.** Prothrombin activation was measured with varying amounts of phospholipids (10% PS, 90% PC) in the absence (A) or in the presence (B) of Factor Va. Activation rates were determined as described under "Materials and Methods". The prothrombin activators tested were Factor Xa (●-●), and the venom activators from Notechis scutatus (□-□), Oxyuranus scutellatus (▲-▲) and Echis carinatus (△-△). Concentrations of protein, present in the final reaction system, were 2 μM prothrombin, 5 nM Factor Va (if present), and 5 nM prothrombin activator, with the exception of the venom activator from Echis carinatus, which was present at 0.625 mU/ml. The rates of prothrombin activation by Factor Xa and Notechis scutatus in the presence of Factor Va and Oxyuranus scutellatus were corrected for the rates in free solution (12, 420 and 69 nM prothrombin activated/min, respectively).

The results show that Factor Xa and the activator from Notechis scutatus gave very low prothrombin activation rates in the absence of Factor Va, even when considerable amounts of phospholipid were present (Fig. 1A). Both activators were stimulated enormously by Factor Va (Fig. 1B), and, under the experimental conditions, prothrombin activation was proportional to the phospholipid concentration, and thus proportional to the number of procoagulant sites present. Prothrombin activation by the Oxyuranus scutellatus activator did not require Factor Va. In the absence of Factor Va (Fig. 1A), high rates of prothrombin activation were observed, that were proportional to the amount of phospholipid present. Addition of Factor Va (Fig. 1B) had no further effect on the activation rates. Prothrombin activation by the purified prothrombin activator from the venom of Echis carinatus was neither affected by phospholipids nor by Factor Va.

From these experiments it is clear that we have the disposal of three different kinds of snake venom prothrombin activators. A Factor Xa-like activator, which is stimulated both by Factor Va and negatively charged phospholipids (Notechis scutatus), an activator that acts like the Factor Xa-Va complex, and which is only stimulated by phospholipids (Oxyuranus scutellatus), and an activator that does not require cofactor components (Echis carinatus). The results also show that the activators from Oxyuranus scutellatus and Notechis scutatus (in the presence of Factor Va) may be useful enzymes to probe phospholipid-like procoagulant activity.

Effect of platelets on prothrombin activation by various snake venom activators. The three snake venom prothrombin activators described above were used to probe the exposure of platelet procoagulant activity, induced by various platelet stimulators. In order to discriminate between Factor Va and surface-effects on prothrombin activation, the rate of prothrombin activation in the presence of $1x10^7$ platelets/ml was measured in the presence as well as in the absence of added Factor Va. The results are summarized in Table I. Prothrombin activation by Echis carinatus, whose venom activator requires no phospholipid or Factor Va, was not influenced by platelets, either in the presence or in the absence of Factor Va. The same prothrombin activation rates were also observed in the presence of platelets stimulated with various platelet activators.

Table I

Effect of platelets in venom-catalyzed prothrombin activation

Platelets (1.7×10^7/ml) were stimulated for 19 minutes with 10 μM ADP, 1 μM A23187, 1.3 nM thrombin, 10 μg/ml collagen or 1.3 nM thrombin plus 10 μg/ml collagen. Prothrombin activation was measured with 1 μM prothrombin in the absence or in the presence of 2 nM Factor Va. The final prothrombin activator concentrations in the assay mixture were 0.2 mU/ml for the venom activator from Echis carinatus, 3 nM venom activator from Notechis scutatus, 1 nM Factor Xa, or 1 nM venom activator from Oxyuranus scutellatus. Rates of prothrombin conversion were determined as described under "Materials and Methods". The activation rates with Factor Xa plus Factor Va, Notechis scutatus plus Factor Va and Oxyuranus scutellatus were corrected for the rates in free solution (0.6, 75 and 10 nM prothrombin activated/min, respectively).

Platelet stimulator	Rate of prothrombin activation (nM prothrombin activated/min)							
	Echis carinatus		Notechis scutatus		Factor Xa		Oxyuranus scutellatus	
	-Va	+Va	-Va	+Va	-Va	+Va	-Va	+Va
None	16.3	16.6	1.4	44	0.3	42	21	22
ADP	16.6	17.5	1.2	26	0.3	47	26	24
thrombin	15.7	16.6	27.2	59	20.9	84	52	69
collagen	15.8	16.0	15.3	102	11.8	463	249	182
collagen + thrombin	15.7	15.5	67.3	165	62.6	694	243	239
A23187	16.1	15.2	24.3	347	35.0	1342	420	461
platelet sonicate	15.3	15.5	45.9	641	28.8	1609	596	625

The phospholipid- and Factor Va-dependent activator from Notechis scutatus shows a remarkable increase of prothrombin-converting activity in the presence of stimulated platelets. The effect of platelets on prothrombin activation by this venom enzyme strongly resembles the platelet effects on Factor Xa-catalyzed prothrombin conversion. The presence of added Factor Va greatly enhanced the prothrombin activation rate, both on stimulated and unstimulated platelets. With added Factor Va, ADP and thrombin seemed to be poor stimulators of platelet procoagulant activity, whereas collagen, or the combination of collagen plus thrombin, induced considerable prothrombin activation rates. The non-physiological calcium ionophore A23187 or a platelet sonicate gave the highest procoagulant activity. In the absence of added Factor Va, these effects were also visible, although much lower rates of prothrombin conversion were found. Here, thrombin alone appeared to be a potent stimulator of platelet procoagulant activity. This effect is presumably due to the participation of platelet Factor V that is released and activated by thrombin, rather than that it is the consequence of any gross rearrangement of the phospholipid distribution of the platelet membranes.

The venom activator from Oxyuranus scutellatus, whose prothrombin-converting activity in model systems is enhanced by phospholipids, but not by Factor Va, is also stimulated by blood platelets. Resting platelets have a relatively low activity in prothrombin activation by the venom from Oxyuranus scutellatus. Activation of the platelets results in rate enhancements that are comparable to the platelet-induced rate enhancements of the Factor Xa-Va-catalyzed prothrombin conversion. These results indicate that platelets stimulate the activator of Oxyuranus scutellatus and the Factor Xa-Va complex in a similar way. Whereas Factor Xa needs Factor Va to bind to platelets and to promote its prothrombin converting activity (3), the Oxyuranus scutellatus activator seems to bind to platelets independent of Factor Va, since prothrombin activation rates in the presence of stimulated and unstimulated platelets are not affected by the presence of Factor Va. This phenomenon makes the Oxyuranus scutellatus activator an interesting tool to further investigate the nature of the platelet procoagulant sites. The prothrombin activators from Notechis scutatus and Echis carinatus were not involved in further experiments. The activator from Notechis scutatus bears so much resemblance with Factor Xa,

that it is unlikely that it will provide additional information about the nature of the procoagulant activity of platelets. The prothrombin activator from Echis carinatus is not affected by platelets and is therefore not suitable to study the procoagulant properties of platelets.

Time course of generation of platelet prothrombin-converting activity probed with Factor Xa-Va and with the venom activator from Oxyuranus scutellatus. The platelet prothrombin-converting activities, determined with various prothrombin activators reported in Table I, were probed after one single platelet activation time (i.e. 19 min.). In a separate experiment we have followed the time course of appearance of platelet procoagulant activity (data not shown). Platelets were stimulated with collagen plus thrombin, and their procoagulant activity was determined after different time intervals by measuring the effect of activated platelets on prothrombin activation by the Factor Xa-Va complex or by the venom activator from Oxyuranus scutellatus. The methodological approach used was identical to that described in Table I.

The procoagulant activity of unstimulated platelets, as probed by both prothrombin activators, was low and did not change in time. Stimulation of the platelets by the combined action of collagen and thrombin resulted in a rapid rise of procoagulant activity. After a few minutes of stimulation, appreciable amounts of prothrombin were converted, both by the Factor Xa-Va complex and the Oxyuranus scutellatus activator. Maximal rates of prothrombin activation were obtained with platelets that were stimulated for 20 minutes or more. For both activators, platelets reached half-maximal prothrombin-converting activity after 8-10 minutes of platelet stimulation, which suggests that the Factor Xa-Va complex and the Oxyuranus scutellatus activator recognize the same platelet procoagulant sites.

Effect of phospholipase A2 on the activity of stimulated platelets in prothrombin activation by Factor Xa-Va or Oxyuranus scutellatus. In a previous paper (11), we have shown that incubation of stimulated platelets with Naja Naja phospholipase A_2, an enzyme that can degrade phospholipid molecules exposed in the outer leaflet of cellular membranes without causing cell lysis, resulted in a dramatic decrease of platelet activity in Factor Xa-Va-catalyzed prothrombin conversion. It was also shown that this

was not due to some adverse effect of phospholipase A_2 on coagulation factors or other platelet functions, and it was argued that the result was evidence for a phospholipid-like nature of the procoagulant sites of platelets stimulated with collagen plus thrombin.

We performed a similar experiment with the prothrombin activator from Oxyuranus scutellatus. Platelets were stimulated for 30 minutes with thrombin plus collagen and were subsequently treated with a small amount of phospholipase A_2. After different time intervals of phospholipase treatment, the remaining platelet procoagulant activity was probed by addition of prothrombin and the Factor Xa-Va complex or Oxyuranus scutellatus. In Fig. 2 it is shown that phospholipase A_2 quickly reduced

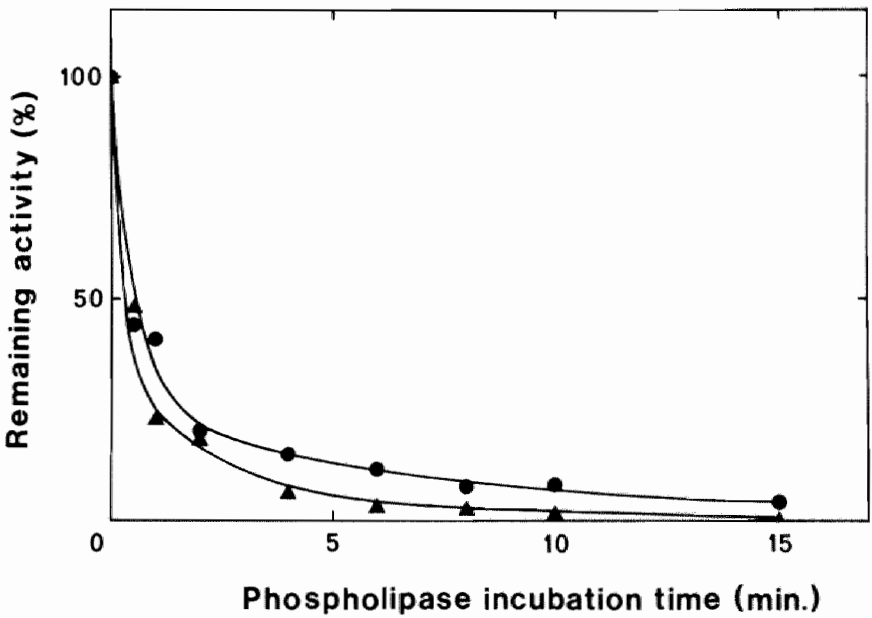

Fig. 2. **Effect of phospholipase A_2 on the activity of stimulated platelets in prothrombin activation.** Platelets (4.3×10^6/ml) were stimulated for 30 minutes with 1.3 nM thrombin plus 10 µg/ml collagen. Subsequently, phospholipase A_2 was added to the activation mixture at a final concentration of 0.02 IU/ml. After the time intervals indicated, the remaining platelet activity in prothrombin conversion was measured as described under "Materials and Methods". Prothrombin activation was determined at 0.5 µM prothrombin and 0.5 nM Factor Xa plus 1 nM Factor Va (●-●), or at 0.5 µM prothrombin and 0.5 nM Oxyuranus scutellatus activator (▲-▲). Prothrombin activation rates obtained with 30-minute stimulated platelets, set at 100%, remained virtually constant when no phospholipase was added.

the procoagulant activity of thrombin plus collagen stimulated platelets, as measured both with the Factor Xa-Va complex and with Oxyuranus scutellatus. This indicates that also in Oxyuranus scutellatus-catalyzed prothrombin activation phospholipids are an important component of the platelet procoagulant sites.

Determination of the number of procoagulant sites on platelet membranes. In an effort to quantitate the number of procoagulant sites on the platelet plasma membrane, experiments were set up to determine two parameters that are essential for sites calculation. The first parameter is the maximal prothrombin activation rate per platelet (Vsat). This maximal rate is obtained when all available procoagulant sites are saturated with the coagulation factors. The second parameter is the maximal rate of prothrombin activation (Vmax) that can be obtained per enzyme complex, bound to the platelet surface. Division of these parameters yields the moles of bound enzyme complexes per platelet from which the number of procoagulant binding sites can be calculated (see legend to Table II). For the determination of Vsat two titrations have to be performed. First, a titration with enzyme at a fixed platelet and prothrombin concentration, in order to determine the enzyme concentration required for saturation of procoagulant sites, is carried out. Subsequently, prothrombin concentrations were varied at the same platelet concentration and at a saturating enzyme concentration, in order to obtain the maximal rate of prothrombin activation per platelet. For the determination of the maximal rate of prothrombin activation per platelet-bound enzyme complex (Vmax) also two titrations were necessary. A titration with platelets (procoagulant sites) at fixed enzyme and prothrombin concentrations was required to determine the platelet concentration at which all enzyme complexes bind to the platelet surface. Then, the concentration of prothrombin is varied under conditions, at which all enzyme is bound to the platelet surface. This titration gives the Vmax of prothrombin activation per platelet-bound enzyme complex.

In principle this method can be applied to determine the sites on non-stimulated platelets as well as on platelets stimulated with various kinds of activators. In Fig. 3 we show a determination of sites on platelets that were stimulated with collagen plus thrombin. The procoagulant sites generated were probed with Factor Xa-Va and with the prothrombin activator

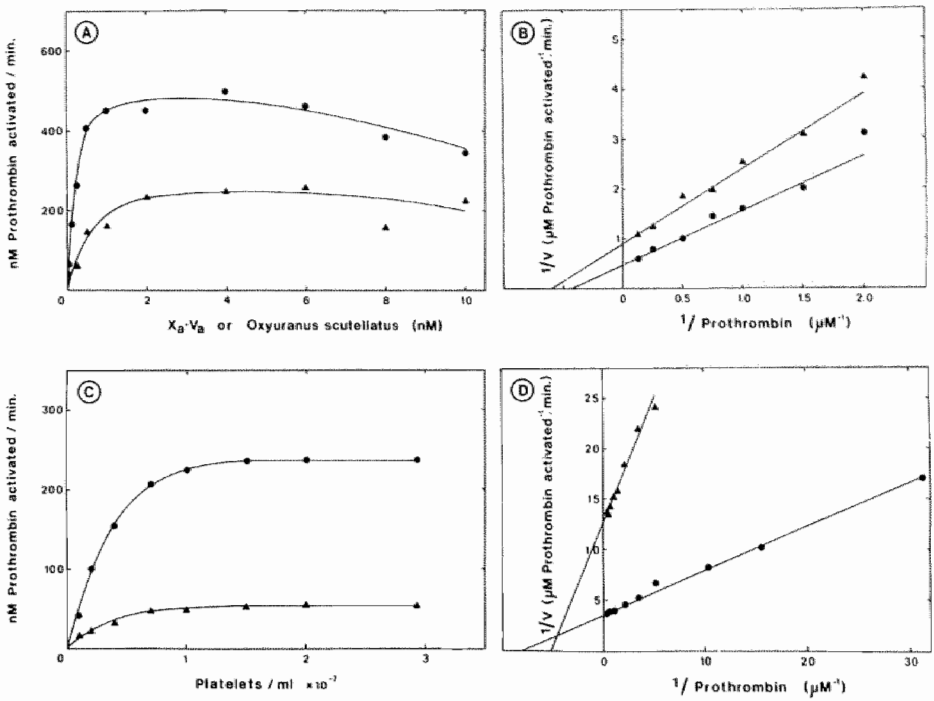

Fig. 3. Determination of the number of procoagulant sites for the Factor Xa-Va complex and the Oxyuranus scutellatus activator on platelet membranes. Platelets were stimulated for 19 minutes with 1.3 nM thrombin and 10 μg/ml collagen. Then, coagulation factors were added and rates of prothrombin activation by Factor Xa plus Factor Va (●-●) or the venom activator from Oxyuranus scutellatus (▲-▲) were measured as described under "Materials and Methods". A) Determination of saturating enzyme concentrations. The concentrations of the Factor Xa-Va complex or the activator from Oxyuranus scutellatus were varied as indicated in the figure. Prothrombin activation was measured at 0.5 μM prothrombin and $1x10^7$ platelets/ml in the final reaction system. B) Determination of the maximal prothrombin activation rate with $1x10^7$ platelets/ml. Rates of prothrombin activation with 5 nM Factor Xa plus 5 nM Factor Va or with 5 nM Oxyuranus scutellatus activator were measured at varying prothrombin concentrations. The results are given in a double reciprocal plot. Kinetic analysis according to Eisenthal and Cornish-Bowden (36) gives Vsat values of $2.09x10^{-16}$ and $1.10x10^{-16}$ moles of prothrombin activated/min/platelet for the Factor Xa-Va complex and Oxyuranus scutellatus activator, respectively. C) Effect of the platelet concentration on prothrombin activation by a limited amount of enzyme. Prothrombin activation was measured with 0.5 μM prothrombin, 0.1 nM Factor Xa and 0.5 nM Factor Va, or with 0.5 μM prothrombin and 0.089 nM Oxyuranus scutellatus activator. D) Determination of the maximal prothrombin activation rate per platelet-bound enzyme complex. Rates of prothrombin activation in the presence of $2x10^7$ platelets/ml were measured at varying prothrombin concentrations. The Factor Xa and Factor Va as well as the Oxyuranus scutellatus concentrations were the same as under (C). Kinetic analysis gives Vmax values of 2822 and 906 nM prothrombin activated/min/nM enzyme for the Factor Xa-Va complex and the Oxyuranus scutellatus activator, respectively.

from the venom of Oxyuranus scutellatus. To determine saturating enzyme concentrations, platelets (1.7×10^7/ml) were stimulated for 19 minutes with collagen plus thrombin and varying amounts of Factor Xa-Va or Oxyuranus scutellatus activator were subsequently added. Finally, prothrombin (0.5 μM) was added to start the reaction. Fig. 3A shows that at concentrations of enzyme > 2 nM saturation of procoagulant sites is achieved. The maximal rate of prothrombin activation with stimulated platelets (1×10^7/ml) at saturating enzyme concentrations (5 nM) was then found by varying the prothrombin concentration. Vsat values were obtained from double reciprocal plots of the rate of prothrombin activation versus the prothrombin concentration (Fig. 3B).

The effect of the concentration of stimulated platelets on prothrombin activation by a limited amount of Factor Xa-Va complex (0.1 nM) or Oxyuranus scutellatus activator (0.089 nM) is shown in Fig. 3C. It appeared that platelets at concentrations above 1.5×10^7/ml did not further increase the reaction rate. Apparently, at 1.5×10^7 platelets/ml, all enzyme is bound to the platelet surface and is involved in prothrombin activation. The maximal rate of prothrombin conversion for 0.1 nM platelet-bound enzyme was subsequently obtained by varying the prothrombin concentration. The result of this experiment, which is given as a double reciprocal plot, is shown in Fig. 3D. From the maximal rates of prothrombin activation determined in Figs. 3B and 3D, it can be calculated that collagen plus thrombin-stimulated platelets expose 44600 sites/platelet for Factor Xa-Va-catalyzed prothrombin activation and 73100 sites/platelet for the Oxyuranus scutellatus-dependent reaction (Table II).

A similar set of titrations was carried out to determine the number of procoagulant sites for the Factor Xa-Va complex and Oxyuranus scutellatus on non-stimulated platelets (data not shown). Non-stimulated platelets expose considerably less procoagulant sites than platelets activated with collagen plus thrombin. For Factor Xa-Va there are 2020 sites/platelet and for the prothrombin activator from Oxyuranus scutellatus there are 3640 sites/platelet (Table II).

In the sites calculation presented above it is assumed that in the determination of the Vmax (Fig. 3D) all added enzyme molecules participate in prothrombin activation. It cannot be excluded, however, that at the limiting amounts of prothrombin activator employed in these experiments, a

Table II

Procoagulant sites on human platelets

The number of functional procoagulant sites was calculated from the formula: number of sites per platelet = Vsat/Vmax x Avogadro's number. Vsat is the rate of prothrombin activation determined with saturating concentrations of activator and prothrombin at non-stimulated platelets (data not shown) or at platelets stimulated with thrombin plus collagen (Fig. 3B). Vmax is the maximal rate of prothrombin activation per molecule of surface-bound activator determined on platelets (Fig. 3D) or obtained from literature (17,33).

Platelet stimulator	sites/platelet[1]		sites/platelet[2]	
	Factor Xa-Va	Oxyuranus scutellatus	Factor Xa-Va	Oxyuranus scutellatus
None	2020	3640	1425	1940
Thrombin + collagen	44600	73100	31460	38970

[1]Calculated with Vmax values determined in Fig. 3D for the Factor Xa-Va complex (2822 min^{-1}) or the venom prothrombin activator from Oxyuranus scutellatus (906 min^{-1}).

[2]Calculated with Vmax values determined on artificial phospholipid vesicles for the Factor Xa-Va complex (4000 min^{-1}, cf. ref. 33) or for the venom prothrombin activator from Oxyuranus scutellatus (1700 min^{-1}, cf. ref. 17).

fraction of the activator molecules may bind to the platelets in a non-specific and non-productive manner (cf. ref 3,8,32). This would result in an underestimation of the Vmax of prothrombin activation. Indeed in model systems in which artificial phospholipid vesicles were used as procoagulant surface, higher Vmax values were observed for the Factor Xa-Va complex (33) as well as for the prothrombin activator from the venom Oxyuranus scutellatus (17). Using the Vmax values reported in literature, a somewhat lower number of sites per platelet was obtained for both the Factor Xa-Va complex and the venom activator from Oxyuranus scutellatus, and there was a closer agreement between the number of sites calculated for both prothrombin activators (Table II).

DISCUSSION

In this paper we present experiments in which a number of snake venom prothrombin activators were used as probes for platelet procoagulant activity. The activity of platelets in prothrombin activation is thought to proceed via binding, interaction and reaction of the coagulation factors at specific sites on the platelet membrane surface. These sites may be composed of either specific protein receptors or negatively charged phospholipid, the latter being less specific in that they will interact with other coagulation factors, such as those of the Factor X-activating complex.

The phospholipid binding site model is supported by the observation that phosphatidylserine residues become exposed on the outer leaflet of the platelet plasma membrane upon platelet activation (10). Since the exposure of phosphatidylserines coincides with an increase of platelet prothrombin- and Factor X-converting activity (6,34), and since the platelets of a patient with a mild bleeding disorder (Scott syndrome) were impaired as well in their ability to expose phosphatidylserine and to generate prothrombin- and Factor X-converting activity (35), it was proposed that phosphatidylserine plays an important role in the binding of the proteins of the prothrombin- and Factor X-activating complexes to the platelet membranes.

In this model also an interaction of platelets with phospholipid-dependent snake venom prothrombin activators can be expected. In this respect the prothrombin activator of Oxyuranus scutellatus may be a potent tool in investigating the nature of the procoagulant sites on the platelet membrane. This venom activator is phospholipid dependent, but does not need Factor Va for procoagulant activity (17). Upon platelet activation, it can therefore probe changes in the platelet membrane phospholipids without measuring any, often confusing, effect of Factor V release and activation.

A number of observations show that Oxyuranus scutellatus probes for the same procoagulant sites on the platelet plasma membrane as the Factor Xa-Va complex. Prothrombin activation by the Factor Xa-Va complex and the Oxyuranus scutellatus activator is similar with respect to the effect of platelets stimulated with various triggers. ADP and thrombin hardly affect platelet procoagulant activity, as probed with both prothrombin activators,

whereas collagen or the combination of collagen plus thrombin are the most potent physiological platelet stimulators. It is shown that the time course of generation of the platelet procoagulant surface, induced by the action of collagen plus thrombin, is the same, irrespective of whether it is determined with Oxyuranus scutellatus or with the Factor Xa-Va complex. Furthermore, the number of procoagulant sites for both activators is approximately the same, both with collagen plus thrombin stimulated and with unstimulated platelets (Table II). Treatment of activated platelets with small amounts of phospholipase A_2 leads to a rapid loss of platelet activity in Factor Xa-Va- and Oxyuranus scutellatus-catalyzed prothrombin conversion.

These results are consistent with phospholipid involvement in the binding of both activators. The explanation of the results presented in this paper does not require postulation of protein receptors that are involved in the binding of the proteins of the prothrombinase complex to the platelet membrane. We feel that our data even argue against the involvement of receptor proteins since it is unlikely that such a receptor, specifically designed for the Factor Xa-Va complex, would also efficiently bind a snake venom protein. Although the Oxyuranus scutellatus activator contains a cofactor which functions like Factor Va (17), it has several properties uncommon with Factor Va. In contrast to Factor Va, the Oxyuranus scutellatus cofactor is not inactivated by EDTA or activated protein C (16). The isolated cofactor part from the venom activator from Oxyuranus scutellatus also lacks the ability to promote prothrombin activation by bovine Factor Xa (17). In summary we conclude that Oxyuranus scutellatus and the Factor Xa-Va complex recognize the same procoagulant sites on both stimulated and non-stimulated platelets and that negatively charged phospholipids are an essential component of these procoagulant sites.

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CHAPTER V

THE EFFECT OF SURFACE CHARGE OF PHOSPHOLIPID VESICLES ON THEIR ACTIVITY IN PROTHROMBIN ACTIVATION

SUMMARY

The conversion of prothrombin into thrombin, which is a crucial reaction in haemostatic plug formation, is greatly stimulated by phospholipids plus calcium ions. It has been proposed that phospholipid surfaces which promote blood coagulation should have a negative surface charge (Bangham, A.D. (1961) Nature 192, 1197-1198). However, the experiments that led to this proposal were carried out with one kind of anionic phospholipid (dicetyl phosphate). Here we report that membranes, which contain phosphatidylserine (PS) as anionic phospholipid, can be made positively charged by incorporation of stearylamine, and still exhibit almost full procoagulant and prothrombin-converting activity. This suggests that electrostatic forces hardly contribute to the binding of coagulation factors to PS-containing membranes. Introduction of stearylamine in membranes containing phosphatidic acid, phosphatidylglycerol, phosphatidylinositol or phosphatidyl-β-lactate (PLac) causes considerable inhibition of their prothrombin-converting activity. Comparison of vesicles containing PS and PLac as anionic phospholipid is of special interest. Since PLac and PS only differ by the presence of an amino group in the polar head group, the much higher procoagulant activity of PS-containing vesicles is indicative for an important function of this amino group of PS in the interaction with coagulation factors. We propose that the association of coagulation factors with PS-containing membranes results from complex formation between Ca^{2+}-ions and ligands supplied by the protein and by PS molecules. The possibility to form such a complex may explain why cell membranes with PS have such excellent procoagulant properties.

INTRODUCTION

The activation of the vitamin K-dependent coagulation factor prothrombin is greatly accelerated by the non-enzymatic protein cofactor Va and by phospholipids plus calcium ions. Kinetic studies have shown that the stimulatory effects by these so-called "accessory components" are due to favourable changes of the kinetic parameters of prothrombin activation, i.e. a decrease of the Km for prothrombin (1-4) and an increase of the Vmax of the reaction (1,5). Although not much is known about the molecular mechanism by which Factor Va enhances the catalytic activity of Factor Xa, there is ample information about the function of phospholipids in the activation of prothrombin. Already in 1967, Jobin and Esnouf (6) and Hemker et al. (7) proposed that the phospholipid-induced acceleration of prothrombin activation was the result of the binding and proper assembly of the participating proteins (prothrombin, Factor Xa and Factor Va) at the phospholipid surface, which facilitate the interactions and hence the reactions between the proteins involved in prothrombin activation. More recent kinetic studies (1-5) support the notion that the enzymatic unit of the prothrombin activator consists of a four component complex (Factor X_a-Factor V_a-calcium-phospholipid) that acts on prothrombin. To obtain optimal rates of activation, prothrombin also has to interact with phospholipids. Indeed, the ability to bind prothrombin, Factor Xa and Factor Va is a prerequisite for procoagulant membranes. In order to associate with the proteins of the prothrombinase complex the membrane should contain negatively charged (anionic) phospholipids. The presence of such phospholipids is essential for the binding of Factor Va (8-10) and for the formation of calcium bridges with the γ-carboxyglutamic acid residues of prothrombin and Factor Xa (11-13).

With respect to the relationship between the procoagulant activity and the chemical and physical properties of the membranes, there are still some unresolved questions. In 1961, Bangham (14) demonstrated with coagulation tests that procoagulant phospholipid bilayers have to possess a net negative charge. This observation was confirmed by Papahadjopoulos et al. (15), who also showed that the procoagulant properties of a membrane mainly depend on the surface charge and not on the chemical structure of its anionic phospholipids. However, more recent reports by Pusey and Nelsestuen (2) and van Rijn et al. (3) indicate that there is no obvious relationship between

the surface charge of procoagulant membranes, their affinity for coagulation factors and their ability to stimulate prothrombinase. Membranes, which contained phosphatidylserine as negatively charged phospholipid, exhibited maximal catalytic efficiency at rather low phosphatidylserine content (2.5 - 10 mol %). It was also shown that membranes with phosphatidylserine were more active than those containing phosphatidic acid although membranes with the latter phospholipid have a higher surface charge. At increasing amounts of negatively charged phospholipid (20-40 mol %) membranes with phosphatidylglycerol, which actually have the lowest affinity for coagulation factors, showed the highest activity in prothrombin activation.

The experiments described in this paper were undertaken, in order to obtain more information on some unanswered questions regarding the effects of the surface charge and the chemical nature of anionic phospholipids on the activity of procoagulant membranes in prothrombin activation.

EXPERIMENTAL PROCEDURES

Reagents. S2238 was purchased from AB Kabi Diagnostica, p-NPGB was from Nutritional Biochemicals. Ovalbumin, Russell's viper venom, Echis carinatus venom and 1,2-dioleoyl-sn-glycero-3-phosphocholine were obtained from Sigma. Phosphatidylinositol was obtained from Koch Light. Column materials for protein purification (DEAE-Sephadex A-50, QAE-Sephadex A-50, SP-Sephadex C-50, Sephadex G-100 and G-200 and Sepharose 4B) were purchased from Pharmacia. Stearylamine (octadecylamine) was from Sigma. Agarose (Isogel agarose-EF) used for the electrophoresis of phospholipid vesicles was obtained from LKB.

Proteins. Bovine prothrombin was purified according to the method of Owen et al. (16). Thrombin was purified as described earlier (1). Bovine Factor X and Factor Xa were prepared according to the method of Fujikawa et al. (17,18). Bovine Factor Va was obtained according to the procedure of Lindhout et al. (19). Proteins were stored at -80 ^{o}C in 50 mM Tris-HCl, 175 mM NaCl and 0.5 mg/ml ovalbumin. Factor Va was stored in the same buffer containing 5 mM $CaCl_2$.

Phospholipids and phospholipid vesicle preparations. 1,2-Dioleoyl-sn-glycero-3-phosphoserine, 1,2-dioleoyl-sn-glycero-3-phosphoglycerol, 1,2-dioleoyl-sn-glycero-3-phosphate and 1,2-dioleoyl-sn-glycero-3-phospho-β-lactate were prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine by enzymatic synthesis as described by Comfurius and Zwaal (20). Single-bilayer phospholipid vesicles were prepared by sonication according to the method of de Kruijff et al. (21). Phospholipid concentrations were determined by phosphate analysis as described by Böttcher et al. (22). The concentration of stearylamine was determined by nitrogen analysis according to Ward et al. (23), using an Antek digital nitrogen detector model 720 coupled to an Antek pyroreactor 771.

Protein concentrations. Active site titration with p-NPGB was used to determine the molar concentrations of thrombin (24) and Factor Xa (25). Prothrombin concentrations were determined by the same method (24) after complete activation of prothrombin with the venom activator from Echis carinatus. The concentration of Factor Va was determined by kinetic analysis as described by Lindhout et al. (19).

Assay system for measuring rates of prothrombin activation. Phospholipids and Factor Xa, either with or without Factor Va, were incubated for 5 min at 37 ^{o}C in a buffer containing 50 mM Tris, 175 mM NaCl, 5 mM $CaCl_2$ and 0.5 mg/ml ovalbumin at pH 7.9. Prothrombin activation was started by the addition of prewarmed prothrombin in the same buffer. After different time intervals, samples from the reaction mixture were transferred to cuvettes containing 235 μM of the thrombin-specific chromogenic substrate S2238 in 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 20 mM EDTA and 0.5 mg/ml ovalbumin. The amount of prothrombin activated in the reaction aliquots was calculated from the absorbance change (ΔA405-500/min), recorded on an Aminco DW-2A spectrophotometer operating in the dual-wavelength mode, and a calibration curve of chromogenic substrate conversion by known amounts of active site-titrated thrombin.

Coagulations tests. Coagulation tests (Stypven time and Activated Partial Thromboplastin Time) were performed as described by Denson (26).

Electrophoresis of phospholipid vesicles. The electrophoretic mobility of phospholipid vesicles was determined by electrophoresis on flatbed agarose gels. The agarose gels were obtained by application of a warm (60 °C) solution of 0.3 % agarose in 50 mM Tris, 175 mM NaCl (pH 7.9) onto a glass plate. The phospholipid vesicles, usually 4 µl of a solution containing 2 mM phospholipid were applied to the agarose gel in small cylindrical holes pierced in the middle part of the gel. Electrophoresis was carried out for 2 h at 15 °C at 3.5 Volt/cm on a LKB 2117 Multiphor electrophoresis apparatus. The electrode buffer, which was the same in the cathode and anode compartments, contained 50 mM Tris, 175 mM NaCl (pH 7.9). During the electrophoresis the electrode buffers were refreshed several times in order to keep their pH and ionic composition constant. After electrophoresis the phospholipid vesicles were stained by incubating the agarose gel in a saturated solution of iodine in water/ethanol (95/5; v/v).

RESULTS

Prothrombin activation on membranes with low phosphatidylserine content. In an earlier publication (3), we have shown that the complete prothrombinase complex (Factor Xa, Factor Va, Ca^{2+}-ions and phospholipid) has a rather low requirement for negatively charged phospholipids, since membranes with low mol percentages phosphatidylserine exhibited the highest catalytic efficiency. Phospholipid vesicles with amounts of phosphatidylserine as low as 2 mol % still maintained favourable kinetic parameters for prothrombin activation. In these experiments, the prothrombinase complex had such a low phosphatidylserine requirement that it even becomes questionable whether prothrombin activation was actually still dependent on the presence of negatively charged phospholipids. However, in Fig. 1 it is shown that membranes have to contain negatively charged phospholipids in order to promote prothrombin activation. In this experiment, phospholipid vesicles with 0, ½, 1, 2, 3, 4 or 5 mol % phosphatidylserine were compared with respect to their ability to stimulate prothrombin activation. When Factor Va is part of the prothrombin-activating complex, membranes containing 3-5 mol % phosphatidylserine showed optimal prothrombin-converting activity. At lower mol percentages of phosphatidylserine there was a gradual decrease of

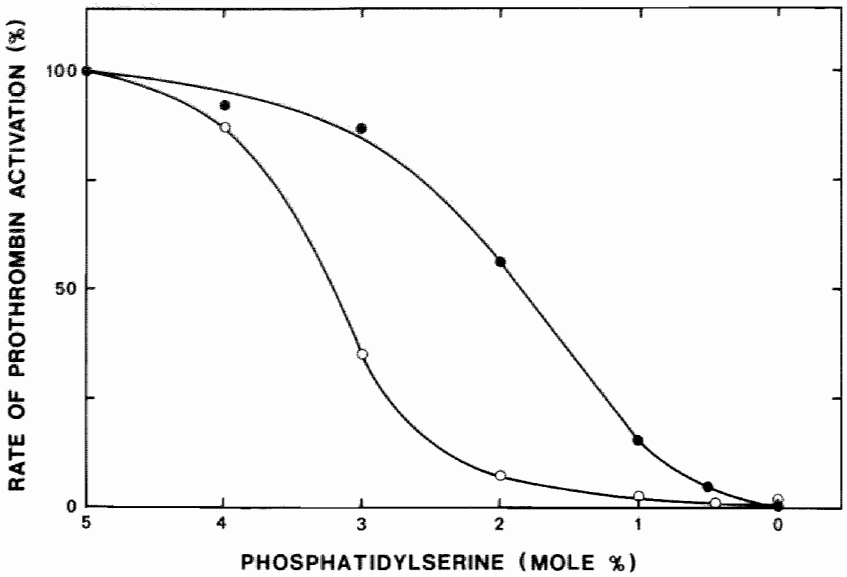

Fig. 1. **Effect of the mol percentage phosphatidylserine on the activity of phospholipid vesicles in prothrombin activation.** Prothrombin (0.5 µM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/ml ovalbumin, 50 µM phospholipid (PS/PC with amounts of PS indicated in the figure), and 1 pM Factor Xa plus 5 nM Factor Va (●-●), or 2 nM Factor Xa (○-○). Rates of prothrombin activation were determined as described in the experimental procedures. 100 % corresponded with rates of 2160 moles prothrombin activated per min per mol Factor Xa (●-●) or 0.17 moles prothrombin activated per min per mol Factor Xa (○-○).

the ability of membranes to promote prothrombin activation. Membranes without phosphatidylserine, which were only composed of the neutral phospholipid phosphatidylcholine, did not show detectable activity in prothrombin activation.

Similar observations were made for prothrombin activation by Factor Xa in the absence of Factor V_a. Under these conditions, vesicles with 5 mol % phosphatidylserine exhibited already diminished prothrombin-converting activity if compared with membranes containing higher mol percentages phosphatidylserine (data not shown). A further decrease of the phosphatidylserine content of the membranes resulted in a considerable decrease of their procoagulant activity. In the absence of Factor Va, membranes with mol

percentages phosphatidylserine below 2 % were not able to stimulate prothrombin activation.

From these experiments, we conclude that, although the prothrombinase complex functions very well on membranes with low amounts of phosphatidylserine, there is still an absolute requirement for the presence of negatively charged phospholipids.

Prothrombin activation on phospholipid vesicles with positive electrostatic potential. In the experiment presented in the previous paragraph, the surface charge of phospholipid vesicles was varied by changing the amount of negatively charged phospholipid (PS). However, in such an experimental set-up, it is not only the surface charge of vesicles that is changed, but there is also a variation of the number of negatively charged phospholipid molecules available for interaction with coagulation factors. To study the effect of the surface charge of phospholipid vesicles on their activity in prothrombin activation (without changing the amount of negatively charged phospholipid) the electrostatic potential of vesicles was varied by introduction of stearylamine in the membrane surface. Stearylamine (CH_3-$(CH_2)_{17}$-NH_2) is a primary amine, with large hydrocarbon chain, that is positively charged at physiological pH (pK $\sim$ 10.5), and which rapidly incorporates into phospholipid bilayers. Since under the experimental conditions phosphatidylserine has a net charge of -1, the introduction of each molecule of stearylamine in a membrane compensates the charge of one phosphatidylserine molecule.

Surprisingly, the incorporation of amounts of stearylamine up to 15 mol % in vesicles with 5 mol % phosphatidylserine hardly affected their activity in prothrombin activation by the complete prothrombinase complex (Fig. 2). Between 0 and 12.5 mol % stearylamine there was no detectable effect on the prothrombin-converting activity of the vesicles. Membranes with 15 mol % stearylamine showed a minor loss of their ability to promote prothrombin activation. Also in coagulation tests (Stypven time or Activated Partial Thromboplastin Time, APTT), vesicles containing 5 mol % phosphatidylserine with or without 15 mol % stearylamine were almost equally effective in shortening the clotting time (data not shown).

Electrophoretic analysis showed that phosphatidylserine-containing vesicles with varying concentrations of stearylamine had electrophoretic

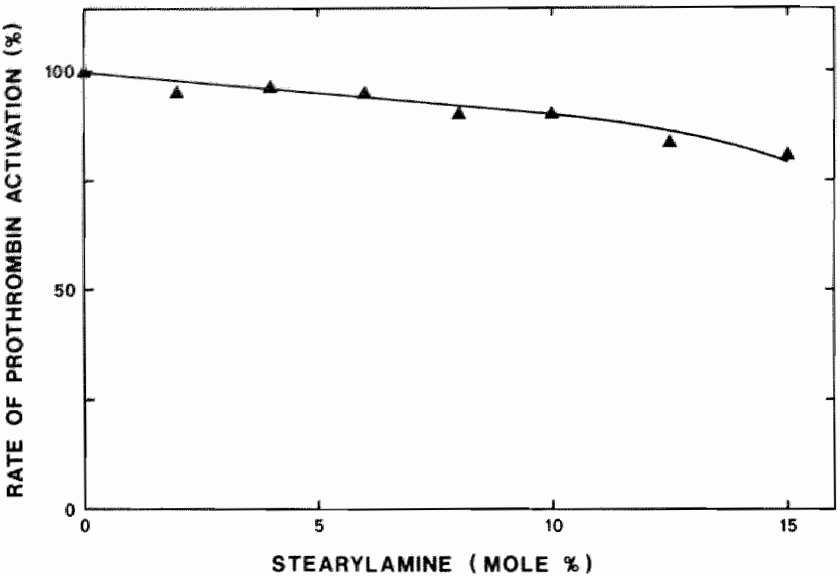

Fig. 2. **Effect of stearylamine on the prothrombin-converting activity of phospholipid vesicles containing 5 mol % phosphatidylserine.** Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/ml ovalbumin, 50 μM phospholipid, 1 pM Factor Xa and 5 nM Factor Va. The phospholipid vesicles contained 5 mol % phosphatidylserine, 95 mol % phosphatidylcholine and amounts of stearylamine indicated in the figure. When stearylamine was present the amount of phosphatidylcholine in the membranes was decreased by 1 % per 3 % stearylamine. Rates of prothrombin activation were determined as described in the experimental procedures. 100 % corresponded with a rate of 2320 moles prothrombin activated per min per mol Factor Xa.

mobilities that corresponded with their calculated surface charge (Fig. 3). Vesicles with amounts of stearylamine less than 6 mol % were negatively charged, since they moved towards the anode, while vesicles with mol fractions of stearylamine exceeding 6 mol % moved towards the cathode which indicates that they had a positive electrostatic potential.

From these experiments it is obvious that membranes active in prothrombin activation do not have to possess a net negative charge. Membranes with positive electrostatic potential are able to promote prothrombin activation provided that they also contain phospholipids with negatively charged head group.

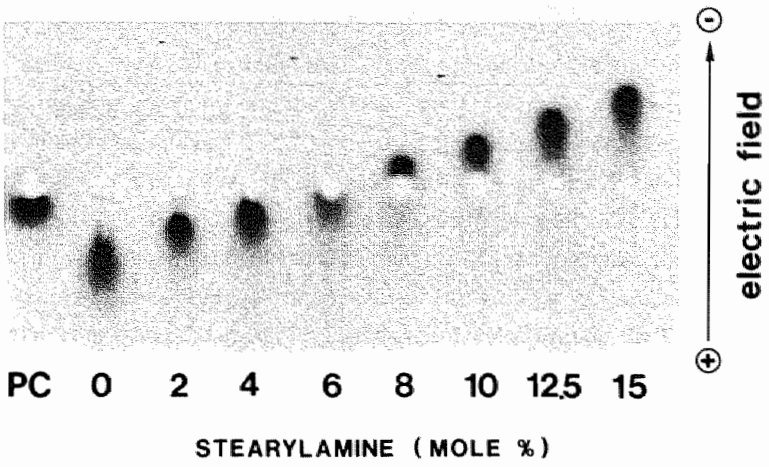

Fig. 3. **Electrophoresis of phospholipid vesicles containing varying amounts of stearylamine.** The phospholipid vesicles used in the experiment presented in Fig. 2 were subjected to agarose gel electrophoresis as described in the experimental procedures. For comparison, non-charged vesicles composed of 100 % phosphatidylcholine (indicated in the figure by PC) were also electrophoresed. After electrophoresis, the vesicles (containing amounts of stearylamine indicated in the figure) were made visible by staining with a saturated solution of iodine in water/ethanol (95/5; w/w).

The effect of stearylamine on the kinetic properties of phosphatidylserine-containing membranes. Since prothrombin activation by the complete prothrombinase complex is already stimulated by membranes with very low phosphatidylserine content (Fig. 1), it has to be ruled out that the procoagulant activity of the phospholipid vesicles with positive electrostatic potential is due to a contamination by a small amount of vesicles with net negative charge. To exclude such an artifact, stearylamine containing vesicles (PS/PC/stearylamine, 5/90/15; mole/mole/mole) were subjected to agarose gel electrophoresis, eluted from the gel, and subsequently compared with non-electrophoresed vesicles and vesicles without stearylamine (PS/PC, 5/95; mole/mole) with respect to their ability to promote prothrombin activation.

Fig. 4 shows the effect of variation of the concentration of different kind of phospholipid vesicles on the rate of prothrombin activation by the

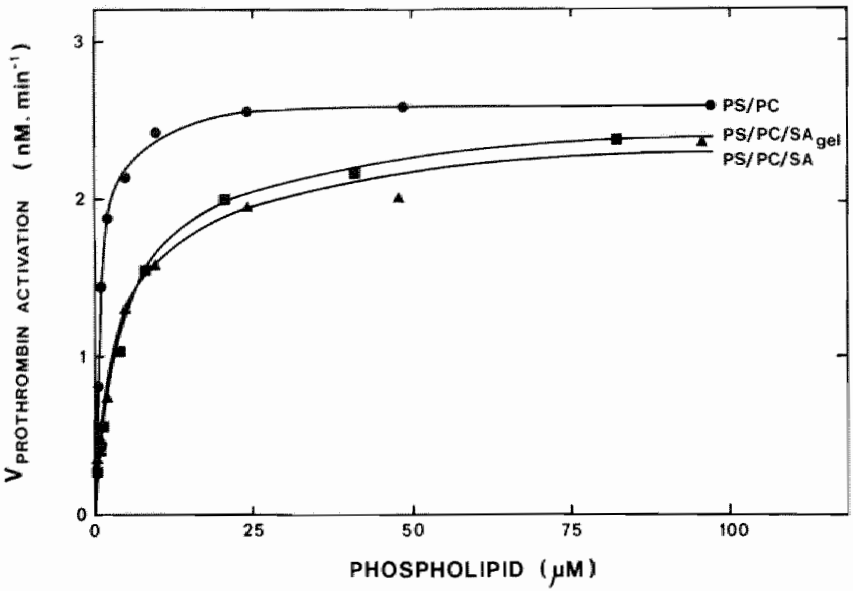

Fig. 4. **Effect of the phospholipid concentration on prothrombin activation by the complete prothrombinase complex.** Prothrombin (2 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/ml ovalbumin, 1 pM Factor Xa, 5 nM Factor Va and amounts of phospholipid indicated in the figure. The phospholipid preparations used in this experiment were: vesicles containing phosphatidylserine and phosphatidylcholine in a molar ratio 5/95 (●-●), vesicles containing phosphatidylserine, phosphatidylcholine and stearylamine in a molar ratio 5/90/15 (▲-▲) and the same vesicles after agarose gel electrophoresis and elution from the gel (■-■). Rates of prothrombin activation were determined as described in the experimental procedures.

complete prothrombinase complex. When procoagulant membranes do not contain stearylamine, rather low concentrations of phospholipid (PS/PC, 5/95; mole/mole) were required to saturate the prothrombin-converting activity of the prothrombinase complex. Half maximal rates of prothrombin activation were obtained at 0.8 μM phospholipid. Upon introduction of 15 mol % stearylamine in the vesicles, considerably more phospholipid was required for saturation of the prothrombinase complex. With stearylamine-containing vesicles, half maximal rates of prothrombin activation were obtained at 4 μM phospholipid. In this experiment, it was not possible to distinguish electrophoresed from

non-electrophoresed stearylamine-containing vesicles, since both phospholipid preparations produced identical saturation curves (Fig. 4).

We also attempted to determine the effect of stearylamine on the number of procoagulant sites present on vesicles. To this end, rates of prothrombin activation were measured at low phospholipid and high prothrombin concentration as a function of the amount of Factor Xa-Va complex. Under such conditions, rates of prothrombin activation are limited by the concentration of phospholipid-bound Factor Xa-Va. From the prothrombin activation rates, obtained at saturating Factor Xa-Va concentrations, one can calculate the concentration of membrane-bound Factor Xa-Va complex (see below).

Fig. 5A shows the effect of various amounts of Factor Xa-Va on the prothrombin-converting activity of vesicles with and without stearylamine. The typical hyperbolic titration curves are indicative for equilibrium

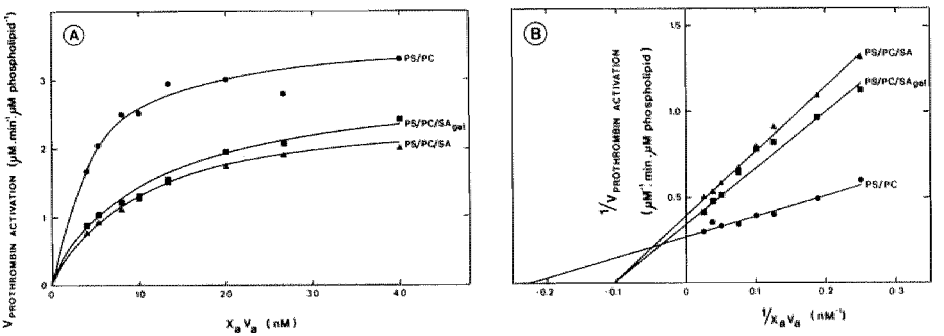

Fig. 5. **Prothrombin activation on various kinds of phospholipid vesicles as function of the Factor Xa-Va concentration.** Prothrombin (4 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/ml ovalbumin, amounts of Factor Xa and Factor Va indicated in the figure, and 0.49 μM phospholipid vesicles (PS/PC, 5/95; mole/mole;●-●), or 0.48 μM phospholipid vesicles (PS/PC/stearylamine, 5/90/15; mole/mole/mole;▲-▲), or 0.82 μM of the same vesicles eluted from an agarose gel after electrophoresis (PS/PC/stearylamine, 5/90/15, mole/mole/mole;■-■). Rates of prothrombin activation were determined as described in the experimental procedures and expressed as μM prothrombin activated/min/μM phospholipid, in order to compare the different phospholipid preparations. (A) direct plots of the rate of prothrombin activation as a function of the concentration of Factor Xa-Va complex. (B) double reciprocal plots of the same data.

saturation of procoagulant sites with the Factor Xa-Va complex. More quantitative information can be obtained from double reciprocal plots of these data (Fig. 5B). The intercepts at the ordinate and abcissa of such plots yield, respectively, the rate of prothrombin activation at saturating concentrations of Factor Xa-Va (Vsat), and the concentration of Factor Xa-Va complex required for 50% saturation of the available procoagulant sites ($K_{\frac{1}{2}}$). From the rate of prothrombin activation at saturating concentrations of Factor Xa-Va one can subsequently calculate the number of functionally active Factor Xa-Va complexes (procoagulant sites) on the phospholipid surface (eq. 1):

$$\text{number of sites} = \text{Vsat/Vmax} \qquad \text{(eq. 1)}$$

in which the number of sites is expressed as moles Factor Xa-Va complex bound per mol of phospholipid, Vsat is the rate of prothrombin activation (μM prothrombin activated/min/μM phospholipid) at saturating Factor Xa-Va concentrations (Fig. 5B) and Vmax is the turnover number of the prothrombinase complex (3000 moles prothrombin activated/min/mol Factor Xa-Va; ref. 3).

Table I summarizes the effect of stearylamine on the "binding parameters" of phospholipid vesicles for Factor Xa-Va. Vesicles without stearylamine (PS/PC, 5/95; mole/mole) were able to bind 1.05×10^{-3} moles of Factor Xa-Va complex per mol of phospholipid with a $K_{\frac{1}{2}}$ of 4.4×10^{-9} M. Introduction of 15 mol % stearylamine in these vesicles hardly affected the number of binding sites and the binding affinity for the Factor Xa-Va complex. There was a small increase of the $K_{\frac{1}{2}}$ to 9.5×10^{-9} M and the number of procoagulant sites was slightly decreased (0.73×10^{-3} moles Factor Xa-Va bound per mol of phospholipid). Stearylamine-containing vesicles that were subjected to gel electrophoresis and that were eluted from the gel had the same binding parameters as the non-electrophoresed vesicle preparation.

From these experiments we conclude that a) the prothrombin-converting activity of membrane preparations with stearylamine (PS/PC/stearylamine, 5/90/15; mole/mole/mole) cannot be due to the presence of a small amount of negatively charged phospholipid vesicles, and b) an excess of stearylamine hardly affects the binding properties of the procoagulant sites on phosphatidylserine-containing membranes.

Table I

Effect of stearylamine on the binding of the Factor Xa-Va complex to phospholipid vesicles

The binding parameters ($K_{\frac{1}{2}}$ and number of binding sites) were obtained from the double reciprocal plots presented in Fig. 5B.

Phospholipid	$K_{\frac{1}{2}}$ (M)	Sites (Xa-Va/phospholipid, mole/mole)
PS/PC (5/95, mole/mole)	4.4×10^{-9}	1.05×10^{-3}
PS/PC/stearylamine (5/90/15, mole/mole/mole)	9.5×10^{-9}	0.73×10^{-3}
PS/PC/stearylamine(gel eluted) (5/90/15, mole/mole/mole)	9.5×10^{-9}	0.84×10^{-3}

Effect of stearylamine on the prothrombin-converting activity of membranes with different anionic phospholipids. In order to test whether the small effects of stearylamine on the prothrombin-converting activity of phospholipid is a unique property of membranes with phosphatidylserine, we also determined the effect of stearylamine on the procoagulant activity of phospholipid vesicles containing other anionic phospholipids. The chemical structures of the anionic phospholipids used in this experiment are presented in Fig. 6. The net charge carried by these phospholipids is different. Phosphatidylglycerol, phosphatidylinositol and phosphatidylserine have a net charge of -1 (phosphatidylserine carries one positive and two negative charges), phosphatidic acid contains either one or two negative charges ($pK_2 \sim 7$), while phosphatidyl-β-lactate carries two negative charges. The latter phospholipid is of particular interest, since it structurally resembles phosphatidylserine. Phosphatidylserine and phosphatidyl-β-lactate only differ by the presence of a NH_2-group ($-NH_3^+$ at physiological pH) in phosphatidylserine.

Table II summarizes the effect of excess of stearylamine (15 mol %) on the prothrombin-converting activity of membranes containing 5 mol % of the various anionic phospholipids. Without stearylamine present, the anionic phospholipids differed already considerably in their ability to promote

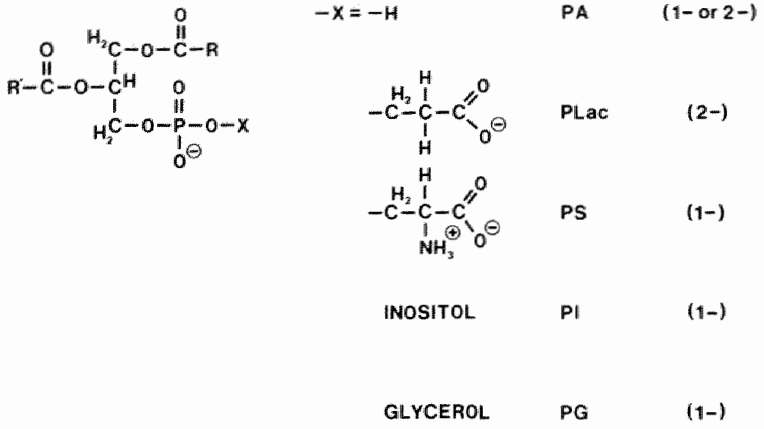

Fig. 6. **Chemical structures of anionic phospholipids.** The prothrombin-converting activities of membranes containing the anionic phospholipids presented in this figure were compared in the experiment described in Table II.

Table II

Effect of stearylamine on the prothrombin-converting activity of membranes containing different anionic phospholipids

Prothrombin (0.5 μM) was activated in a reaction mixture containing 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 5 mM $CaCl_2$, 0.5 mg/ml ovalbumin, 50 μM phospholipid, 5 pM Factor Xa and 5 nM Factor Va. Vesicles without stearylamine contained 95 mol % PC and 5 mol % anionic phospholipid; vesicles with stearylamine contained PC, anionic phospholipid and stearylamine in a molar ratio 90/5/15. Rates of prothrombin activation were determined as described in the experimental procedures.

Phospholipid	Prothrombin activation (nM/min)		Inhibition (%)
	-stearylamine	+stearylamine	
PS/PC	10.6	8.8	17
PA/PC	6.8	1.1	84
PLac/PC	2.3	0.21	91
PG/PC	0.36	0.022	94
PI/PC	0.29	0.017	94

prothrombin activation. The procoagulant activity of the membranes decreased in the order phosphatidylserine > phosphatidic acid > phosphatidyl-β-lactate > phosphatidylglycerol > phosphatidylinositol. Upon incorporation of stearylamine all membranes retained at least part of their activity in prothrombin activation. However, the effects of stearylamine on the prothrombin-converting activity was much more pronounced on membranes containing anionic phospholipids other than phosphatidylserine. Vesicles with phosphatidic acid were inhibited by stearylamine for some 80%, while the incorporation of stearylamine in vesicles with phosphatidyl-β-lactate, phosphatidylglycerol or phosphatidylinositol inhibited their prothrombin-converting activity more than 90%. Apparently, stearylamine has more effect on the prothrombin-converting activity of membranes, which by itself already have a decreased activity in prothrombin activation and a low affinity for coagulation factors (27).

Table II contains a few other observations that need further attention. The data presented in this table show that there is no direct relation between the procoagulant activity of membranes and the surface charge provided by their anionic phospholipids. Vesicles containing 5 mol % phosphatidyl-β-lactate or 5 mol % phosphatidic acid, which have a higher surface charge than vesicles with 5 mole % phosphatidylserine are less active in prothrombin activation. In this respect the difference between phosphatidyl-β-lactate and phosphatidylserine may be of special interest. Since these phospholipids only structurally differ by the presence of an amino group in phosphatidylserine, their different procoagulant activities are indicative for an important function of this amino group in the interactions with the proteins of the prothrombinase complex.

DISCUSSION

The results obtained in this study provide information that will be relevant to understand the chemical and physical requirements of phospholipid membranes, which promote the activity of the prothrombinase complex. In this paper it is shown that a) such membranes have to contain negatively charged phospholipids, b) there is no obvious relation between the surface charge of membranes and their activity in prothrombin activation, c) there

is no requirement for a negative surface charge, since membranes with positive electrostatic potential exhibit considerable prothrombin-converting activity, and d) membranes with phosphatidylserine are much more active than membranes with phosphatidyl-β-lactate, which is indicative for an important function of the amino group of phosphatidylserine in the interaction with coagulation factors.

Conflicting views exist as to the chemical and physical nature of the calcium-dependent interaction between vitamin K-dependent coagulation factors and the negatively charged head groups of anionic phospholipids in procoagulant membranes. Dombrose et al. (28) reported that the binding of prothrombin fragment 1 (the γ-carboxyglutamic acid-containing activation fragment of prothrombin) to phospholipid vesicles composed of phosphatidylglycerol and phosphatidylcholine is highly ionic strength dependent. Based on this observation they proposed that electrostatic interactions significantly contribute to protein-membrane association. In a hypothetical model the binding of prothrombin fragment 1 to membranes was visualized as follows. Prothrombin fragment 1 itself is negatively charged. When calcium-ions bind to the γ-carboxyglutamic acid residues of fragment 1, it becomes positively charged and will be electrostatically attracted by the negatively charged phospholipid surface. The association of prothrombin fragment 1 with calcium ions (i.e. its change from negative to positive charge) is promoted by the fact that in the vicinity of the negatively charged phospholipid surface there is a calcium-ion gradient with greatly increased local calcium concentration near the membrane surface.

Resnick and Nelsestuen (29) showed that the binding of prothrombin to membranes containing phosphatidylserine and phosphatidylcholine (PS/PC, 20/80; mole/mole) was rather insensitive to variation of pH and ionic strength. Based on this observation they proposed a chelation model for prothrombin-membrane binding. In this model, calcium ions are thought to form a coordination complex with the two carboxylgroups of γ-carboxyglutamic acid in prothrombin and two negative charges supplied by anionic phospholipids of the membrane surface.

We feel that our results are consistent with a chelation model. The observation that a change of the surface charge of phosphatidylserine-containing vesicles from a negative to a positive potential hardly affects their activity in prothrombin activation, indicates that in this case

electrostatic interactions have little contribution to protein-membrane association. It cannot be ruled out, however, that such interactions may be more important for protein binding to membranes containing anionic phospholipids like phosphatidic acid, phosphatidyl-β-lactate, phosphatidylglycerol or phosphatidylinositol. Considering the chelation model to be applicable to membranes with phosphatidylserine we propose that the amino group of this phospholipid functions as a ligand in the chelate complex. Assuming the participation of a hypothetical ligand X, for instance supplied by the protein, it becomes possible to obtain an octahedral structure (cf. Ca-EDTA complex) with the two carboxyl groups of γ-carboxyglutamic acid and the phosphate, amino and carboxyl groups of phosphatidylserine (Fig. 7). It is likely that such a complex with 6 coordination bonds has an increased stability which can explain why membranes with phosphatidylserine have such excellent procoagulant properties. This may also have physiological significance since phosphatidylserine is the major anionic phospholipid in blood platelets and endothelial cells, the membranes of which are thought to stimulate coagulation in situ.

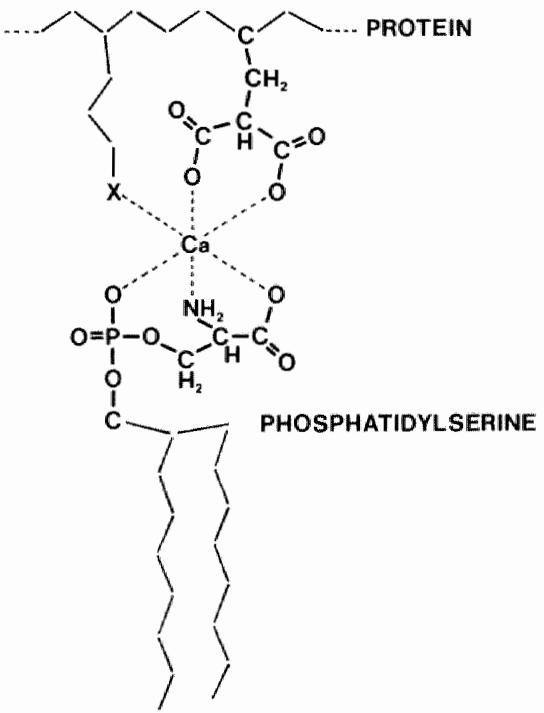

Fig. 7. Hypothetical model for chemical interactions involved in Ca^{2+}-dependent binding of vitamin K-dependent proteins to procoagulant membranes.

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CHAPTER VI

GENERAL DISCUSSION

The conversion of prothrombin to thrombin by Factor Xa is a very slow process which is accelerated enormously in the presence of a phospholipid surface, calcium ions and the protein cofactor Factor Va. Kinetic experiments have shown that phospholipids exert their effect by drastically decreasing the Km for prothrombin, while the major effect of Factor Va is a thousand-fold increase of the k_{cat} of prothrombin activation. On membranes with a low affinity for prothrombin, Factor Va also causes an additional decrease of the Km for prothrombin. Little information on the molecular interactions responsible for these effects is, however, available. In this thesis experiments are described that aim to further elucidate the interactions of the components involved in prothrombin activation.

Prothrombin conversion proceeds by phospholipid-bound Factor Xa or, if Factor Va is present, by phospholipid-bound Factor Xa-Va complex. It appears that prothrombin activation in the absence or presence of Factor Va cannot be described by a single model (1). Without Factor Va, the density of prothrombin bound at the phospholipid surface determines the reaction kinetics (bound substrate model), while in the presence of Factor Va the reaction rate is governed by the prothrombin concentration in free solution (free substrate model).

The experiments presented in chapter II are consistent with these observations. In the absence of Factor Va, the prothrombin fragments, fragment 1 (F 1) and fragment 1.2 (F 1.2), are efficient inhibitors of prothrombin activation. The fragments apparently compete with prothrombin and Factor Xa for binding sites at the phospholipid surface, which will result in reduction of the amounts of enzyme and substrate participating in prothrombin conversion. These results can be easily explained in the bound substrate model. However, when Factor Va is present, the activation peptides are much less effective in reducing the rate of prothrombin conversion. This result is consistent with the view that Factor Va promotes

the assembly of the prothrombinase complex through interactions with Factor Xa and prothrombin that are independent of their membrane binding domain, which explains why the fragments do not interfere with these interactions. In some experiments the prothrombin fragments even stimulate the reaction, indicating that with the complete prothrombinase complex the free substrate model is indeed applicable. Recently, it was shown by Forman and Nemerson (2) that prothrombin fragment 1 also stimulates the activation of Factor X via the tissue factor pathway. This observation was also taken as support for a mechanism in which soluble substrate (in this case Factor X) directly interacts with a surface-bound catalytic unit.

Two models have been proposed to explain the observation that Factor Va considerably decreases the Km for prothrombin activation on membranes with a low affinity for prothrombin. A Factor Va induced clustering of phosphatidylserine molecules, as reported by Mayer and Nelsestuen (3), may create a better surface for prothrombin binding and hence causes a decrease of the Km. We would expect, however, that with such a mechanism both F 1 and F 1.2 would be efficient inhibitors of the complete prothrombinase complex on low affinity membranes, which is apparently not the case. It is also possible that Factor Va promotes binding of prothrombin to the prothrombinase complex by direct interaction with the prothrombin fragment 2 region (4). Such an enhanced binding affinity can be correlated with the Factor Va-induced decrease of the Km for prothrombin. This is, however, not supported by our results, since in none of the experiments F 1.2 is a better inhibitor than F 1. Because none of the existing hypotheses fits our data an alternative explanation for the effect of Factor Va on the Km for prothrombin is given in chapter II. It is known that Factor Va increases at least one limiting forward rate constant of prothrombin activation (5). Since the Km for prothrombin is a complex function of dissociation constants and forward rate constants, we propose that the raise of a rate constant may also decrease the Km for prothrombin. In that case it is not to be expected that the activation fragments will inhibit prothrombin activation, since they can only interfere with direct binding interactions and not with the individual steps in the reaction mechanism.

More information on the mechanism of prothrombin activation and the role of the accessory components can be obtained by studying snake venom prothrombin activators. Snake venom activators can have widely different

structural and functional properties and may, therefore, add to our knowledge of the mode of action of the physiological activator, Factor Xa. In Chapter III we describe the purification and characterization of the prothrombin activator from the venom of Oxyuranus scutellatus. The venom activator was purified by gel filtration and ion-exchange chromatography. The enzyme has a molecular weight of about 300,000 and consists of subunits of M_r 110,000, 80,000 and two disulfide-linked polypeptides of M_r 30,000. One or both of the M_r 30,000 subunits contain the active site. Prothrombin activation by the venom enzyme is greatly stimulated by phospholipids plus calcium ions, but is not affected by the presence of Factor Va. Several experiments indicate that the activator has many properties in common with the Factor Xa-Va complex, and suggest that the enzyme is a multimeric protein, consisting of a catalytic unit with Factor Xa-like activity and a cofactor part, which functions like Factor Va. The catalytic unit is probably formed by the two M_r 30,000 subunits, while the cofactor consists of the M_r 110,000 and 80,000 subunits.

The forces responsible for the interaction of the catalytic domain and the cofactor part are not yet clear. It is possible that hydrophobic interactions are important, since treatment of the activator with NaSCN results in a rapid loss of prothrombin-converting activity, while amidolytic activity towards the chromogenic substrate S2337 remains unaffected. Bovine Factor Va can partly restore the activity of the NaSCN-treated activator preparation, indicating that either a separation of the two functional domains of the activator (i.e. catalytic and cofactor unit) or denaturation of the cofactor part did occur upon NaSCN-induced inactivation.

We have as yet no information on the binding interaction of the venom protein with phospholipids. It is, however, interesting to mention that the venom activator contains γ-carboxyglutamic acid residues which may be involved in the calcium-mediated binding of the enzyme to phospholipid surfaces.

Finally, amino acid sequence analysis of the activator and comparison with known sequences of Factor Xa and Factor Va should reveal if the venom activator indeed shares significant sequence homology with these proteins. If homologies exist, such a study may give relevant information on functional domains in the mammalian proteins.

The purified prothrombin activator from Oxyuranus scutellatus was employed in studies on the properties of the platelet procoagulant sites. Platelets, particularly if activated, have the ability to promote the assembly of the prothrombin- and Factor X-activating complexes and thus enhance the rate of coagulation factor activation. The chemical nature of the procoagulant sites is still a matter of debate: they may either consist of specific receptor proteins or be composed of anionic platelet phospholipids. With respect to the last possibility an important function of phosphatidylserine in the procoagulant sites has been suggested. This phospholipid is located almost exclusively in the cytoplasmic leaflet of the plasma membrane of resting platelets. Upon stimulation of the platelets with collagen plus thrombin the phosphatidylserine is thought to be translocated by a so-called "flip-flop" mechanism to the outside half of the membrane, thereby raising the procoagulant activity of the platelets (6).

In chapter IV it is shown that platelets also promote the prothrombin-converting activity of the venom activator from Oxyuranus scutellatus. When platelets are stimulated with collagen plus thrombin they are about twenty times more active than unstimulated platelets in supporting venom-catalyzed prothrombin activation. A number of observations indicate that the venom activator and the Factor Xa-Va complex recognize the same procoagulant sites on the platelet surface. The venom activator and Factor Xa-Va complex equally well probe the generation of a procoagulant surface upon platelet stimulation with various triggers. Also the time course of appearance of platelet prothrombin-converting activity is identical for both activators. Furthermore, the number of procoagulant sites on the platelet surface is about the same for the venom enzyme and Factor Xa-Va complex. The involvement of platelet phospholipids is emphasized by the effect of phospholipase A_2 treatment of the platelets. Incubation of platelets with phospholipase A_2 causes degradation of phospholipids exposed at the outside of the membrane and leads to a rapid decrease of platelet procoagulant activity as probed with Factor Xa-Va or with venom enzyme. These results argue against the involvement of specific receptor proteins in the assembly of prothrombinase complex at the platelet surface, since it is unlikely that the venom prothrombin activator would recognize such a receptor. We are aware that this evidence is not conclusive since it cannot be excluded

that the venom cofactor part may also have affinity for the proposed protein receptor site. It should be stressed, however, that the participation of protein receptors is not necessary to explain our observations in kinetic experiments. Anionic phospholipids exposed in the outer leaflet of the platelet plasma membrane can equally well provide functional binding sites for a prothrombin-activating complex consisting of either Factor Xa-Va or venom enzyme.

The experiments reported in chapter V are indicative for a special role of phosphatidylserine (PS) in prothrombin activation. Phosphatidylcholine (PC) vesicles containing small amounts of PS provide an excellent procoagulant surface, while other negatively charged phospholipids, such as phosphatidic acid (PA), phosphatidyl-β-lactate (PLac), phosphatidylglycerol (PG) and phosphatidylinositol (PI), are less effective. Furthermore, the activity of vesicles with those anionic phospholipids is very sensitive to variations of the surface charge. Incorporation of positively charged stearylamine in the vesicles, which reverses their surface charge, greatly affected prothrombinase activity, while such a charge reversal hardly affected the activity of PS-containing membranes. This result suggests that the prothrombinase activity of membranes which contain PS as anionic phospholipid is relatively independent of the surface charge of the vesicles. This seems to be in contrast with the experiments of Bangham (7) who demonstrated with clotting assays that a negative surface potential is an absolute requirement for procoagulant activity. However, he used mixtures of PC and dicetylphosphate, an anionic lipid that structurally resembles PG and PI in having one negative charge on the phosphate group and lacking other functional groups for interaction with calcium and/or coagulation factors. Therefore, it is possible that electrostatic interactions form an important contribution to the binding of coagulation factors to phospholipids of this kind. Resnick and Nelsestuen (8) showed that prothrombin binding to PS/PC membranes is relatively insensitive to changes in ionic strength and pH when both the protein and the membrane are saturated with calcium. They suggested that prothrombin-membrane interaction is accomplished by the formation of a mixed chelate complex in which calcium is coordinated by ligands supplied by the protein and the membrane. Our results with PS/PC vesicles also show the importance of non-ionic interactions and are, therefore, consistent with a chelation

model. Comparison of the activity of PS and PLac, which differ only by the presence of a primary amine in the polar part of PS, indicates that the amino group of PS may have an important function in the formation of such a coordination complex. We propose that the amino group, together with the phosphate and carboxyl group, acts as a ligand to stabilize the complex. The possible participation of the amino group, which at neutral pH exists in the protonated form, is indirectly supported by the observation that complexation of calcium by PS molecules results in the release of H^+ (9,10). Completion of the coordination sphere of the Ca^{2+} ion can be obtained with ligands supplied by the protein. The formation of an octahedral complex may involve two carboxyl groups of a Gla residue and an amino group of a basic amino acid residue, several of which are present in the membrane-binding domain of vitamin K-dependent proteins.

It is, however, not necessary that all Gla residues of vitamin K-dependent coagulation factors are directly involved in protein-membrane association. Some of the Gla residues participate in the stabilization of a calcium-induced conformational change of the proteins, which is a prerequisite for protein-membrane interaction (11). Prothrombin and prothrombin fragment 1 contain two or three high affinity calcium binding sites and several sites with lower affinity (12,13). The high affinity sites consist of Gla residues that form an intramolecular bridge through a calcium ion (14). Occupation of these sites is necessary for the protein transition which may subsequently render a line-up of lower affinity Gla-defined calcium binding sites, suitable for membrane interaction. Therefore, the function of the Gla residues seems to be twofold. Some of them are involved in a protein transition by complexing Ca^{2+} ions, while others may directly participate in membrane binding by forming a mixed calcium chelate together with ligands from the membrane (15). The concept of two classes of metal-binding sites (16) is supported by the following observations. The protein conformational change can be induced by several divalent ions other then calcium, especially Mg^{2+}, Mn^{2+} and Sr^{2+} ions are effective (17). Protein-membrane binding, rendering a functional active protein is, however, only supported by Ca^{2+} ions and, to a lesser extent, by Sr^{2+} and Ba^{2+} ions, while Mg^{2+} and Mn^{2+} do not support association (17). The distinct cation specificity may reflect differences in geometry of the two types of metal ion binding sites (cf. ref. 18).

The majority of the prothrombin activation studies described in chapter V were carried out with the complete prothrombinase complex and thus give only partial information on the individual protein-membrane interactions. The results, however, suggest that variation of the vesicle surface charge has minor effects on the membrane affinity for Factor Xa and prothrombin, but also for Factor Va. For the latter protein this seems to be inconsistent with the observation that Factor Va binding is highly ionic strength dependent and is therefore thought to be electrostatic in nature. However, experiments by Pusey et al (19) show that variation of the ionic strength and of the PS content of the membrane only affects the dissociation of the Factor Va-membrane complex, but has a negligible effect on the association between Factor Va and the membrane. These results led to a model that assumes that non-ionic forces are important for the initial association of Factor Va with the membrane. Stabilization of the binding interaction presumably occurs by a subsequent clustering of PS molecules around the protein (3) which results in a further electrostatic attraction between a positively charged domain of Factor Va and a highly negatively charged site at the membrane. Such a model can also explain why Factor Va can bind to PS-containing surfaces with net positive charge. Also on these surfaces initial non-ionic forces leading to membrane-protein association can be followed by the generation of PS-rich domains at the protein-lipid interface, that stabilize the Factor Va-membrane complex.

Data are, however, lacking at this time to support these views. An extension of the kinetic studies and experiments to elucidate individual protein-membrane interactions at net positively charged surfaces may give valuable information as to which interactions are actually important for proper functioning of the prothrombinase complex.

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SUMMARY

In this thesis a study on the mechanism of the Factor Xa-catalyzed conversion of prothrombin to thrombin is described. Prothrombin activation is studied both in model systems with purified phospholipids, as well as in the presence of blood platelets, the blood cells which under physiological conditions are thought to provide the procoagulant surface.

During the conversion of prothrombin, the activation peptides fragment 1 and fragment 1.2 are generated. These peptides contain the membrane binding domain of prothrombin. They have the ability to interfere with the association of the various constituents of the prothrombinase complex with the membrane and are therefore potential inhibitors of thrombin formation. The experiments presented in chapter II show that both fragments indeed effectively inhibit prothrombin activation at the membrane surface. However, Factor Va protects against inhibition, indicating that in the presence of this cofactor prothrombin conversion proceeds via a different mechanism.

Prothrombin activation may also be catalyzed by snake venom proteins. In chapter III the purification and characterization of the prothrombin activator from the venom of Oxyuranus scutellatus (Taipan snake) is described. The prothrombin activator present in the venom of this snake is a multimeric protein consisting of a catalytic unit, showing similarities with Factor Xa, and a cofactor part, which functions like Factor Va. Prothrombin activation by the venom activator is greatly enhanced by phospholipids plus calcium, but not by Factor Va.

The snake venom activator is subsequently used to characterize the procoagulant sites on the platelet surface. Human platelets stimulate the activity of the enzyme. The results presented in chapter IV suggest that Factor Xa-Va complex and the snake venom activator recognize the same procoagulant sites on the platelet surface and that anionic phospholipids play an important role in the interaction of blood platelets with coagulation factors as well as with the snake venom activator.

The chemical and physical properties of procoagulant membranes were further studied by determining the effect of variation of the surface charge and kind of anionic lipid on the procoagulant activity of

phospholipid vesicles (chapter V). Only if membranes contain phosphatidylserine as anionic lipid, the prothrombin-converting activity appears to be virtually independent of the surface charge of the vesicles. Vesicles with a net positive charge (obtained by introduction of stearylamine) exhibit procoagulant activity that is almost equal to that of vesicles with a net negative charge. The results support a chelation model for the calcium-dependent association of coagulation factors with membranes that contain phosphatidylserine as procoagulant phospholipid.

SAMENVATTING

In dit proefschrift wordt een onderzoek beschreven naar het mechanisme van de door Factor Xa gecatalyseerde omzetting van prothrombine in thrombine. De prothrombine-activering is zowel bestudeerd in modelsystemen met gezuiverde fosfolipiden, als in aanwezigheid van bloedplaatjes, de bloedcellen, die onder fysiologische omstandigheden geacht worden het procoagulante membraan te leveren.

Tijdens de omzetting van prothrombine worden de activatiepeptiden fragment 1 en fragment 1.2 gevormd. Deze peptiden bevatten het membraanbindend domein van prothrombine. Ze kunnen interfereren met de associatie van de verschillende componenten van het prothrombinasecomplex met de membraan en zijn daarom potentiële remmers van de vorming van thrombine. De experimenten in hoofdstuk II laten zien dat beide fragmenten de prothrombine-activering op het membraan inderdaad effectief remmen. Factor Va beschermt echter tegen remming, wat aangeeft dat in aanwezigheid van deze cofactor de prothrombine-omzetting via een ander mechanisme verloopt.

Prothrombine-activering kan ook gecatalyseerd worden door slangegifeiwitten. In Hoofdstuk III wordt de zuivering en karakterisering van de prothrombine-activator uit het gif van Oxyuranus scutellatus (de Taipanslang) beschreven. De prothrombine-activator aanwezig in dit gif is een multimeer eiwit dat bestaat uit een catalytisch deel, dat overeenkomsten heeft met Factor Xa, en een cofactor, die functioneert als Factor Va. Prothrombine-activering door de gifactivator wordt aanzienlijk versneld door fosfolipiden plus calcium, maar niet door Factor Va.

De slangegifactivator is vervolgens gebruikt om de procoagulante plaatsen op het oppervlak van bloedplaatjes te karakteriseren. Humane bloedplaatjes blijken de werking van het enzym te stimuleren. De resultaten gepresenteerd in hoofdstuk IV suggereren dat het Factor Xa-Va complex en de slangegifactivator dezelfde procoagulante plaatsen op bloedplaatjes herkennen en dat negatief geladen fosfolipiden een belangrijke rol spelen bij de interactie van bloedplaatjes met zowel de stolfactoren als met de slangegifactivator.

De chemische- en fysische eigenschappen van stolactieve membranen zijn nader bestudeerd door bepaling van het effect van variatie van de oppervlaktelading en variatie van het soort negatief geladen lipide op de

procoagulante activiteit van fosfolipide vesicles (hoofdstuk V). Alleen indien membranen fosfatidylserine als negatief geladen lipide bevatten blijkt de oppervlaktelading geen belangrijke parameter te zijn voor de prothrombinase-activiteit. Vesicles met een netto positieve lading (verkregen door incorporatie van stearylamine) vertonen een procoagulante activiteit die vrijwel gelijk is aan die van vesicles met een netto negatieve lading. Deze resultaten ondersteunen een chelatiemodel voor de calcium-afhankelijke associatie van stolfactoren met membranen, die fosfatidylserine als procoagulant fosfolipide bevatten.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 16 december 1957 geboren te Eindhoven. Na het behalen van het diploma Atheneum B werd in 1976 aangevangen met de studie scheikunde aan de Rijksuniversiteit Utrecht. In december 1979 behaalde hij het kandidaatsdiploma. In januari 1984 werd het doctoraal examen afgelegd met als hoofdvak biochemie (Prof. Dr. J. de Gier) en als bijvak fysiologische chemie (Prof. Dr. Ir. J.S. Sussenbach). Van juli 1984 tot juli 1987 was hij als medewerker van ZWO/MEDIGON werkzaam bij de vakgroep Biochemie aan de Rijksuniversiteit Limburg, waar onder leiding van Prof. Dr. R.F.A. Zwaal en Dr. J. Rosing het promotie-onderzoek werd uitgevoerd, dat geleid heeft tot deze dissertatie. Van juli 1987 tot januari 1988 is hij als toegevoegd onderzoeker verbonden aan de Rijksuniversiteit Limburg.